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Toxicity of manufactured particulate materials on plant root growth

Ling Yang
New Jersey Institute of Technology

ABSTRACT

TOXICITY OF MANUFACTURED PARTICULATE MATERIALS ON PLANT ROOT GROWTH

by
Ling Yang

The rapid development of particle technology and the growing use of particulate materials in industries are bringing large amounts of manufactured particles into the environment. Epidemiological studies suggest that fine particles (particles with aerodynamic diameter smaller than 2.5 μm, i.e., $PM_{2.5}$) have an association with various adverse health effects in humans. Mass studies have been performed on the toxicity and toxicological mechanisms of airborne particles such as $PM_{2.5}$ and PM_{10}, but there are very few investigations which contribute to the knowledge base on biological implications of manufactured particulate materials. Up to now, the published toxicity studies on manmade particulate materials focus on human health effects. No investigations have addressed to the ecological effects of the particulate materials. Toxicities of manufactured particles are evaluated by means of a root elongation test in this study. The particles studied include 13-nm alumina, 14-nm hydrophilic silica, 21-nm titania, 161.2-nm spherical hydrophilic silica, 1.0-μm alumina, 667.6-nm spherical hydrophilic silica, and 0.96-μm titania. Six plant species, *Zea mays* (corn), *Cucumis sativus* (cucumber), *Avena sativa* (oat), *Glycine max* (soybean), *Brassica oleracea* (cabbage), and *Daucus carota* (carrot) were used in this study of the phytotoxicity of the commercially available manufactured particles. Physical and chemical characterization techniques of FTIR, SEM/EDS, the BET method, and particle size analysis, as well as liquid phase coating techniques were applied simultaneously to facilitate the study on toxicological

mechanisms of these manufactured particles. The results indicate that phytotoxicity of particles does not depend solely on the particle mass concentration, particle chemical composition, particle size, as well as the particle specific surface area. It also depends on particle surface characteristics.

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by
Ling Yang

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APPROVAL PAGE

TOXICITY OF MANUFACTURED PARTICULATE MATERIALS ON PLANT ROOT GROWTH

Ling Yang

Dr. Daniel J. Watts, Dissertation Advisor — Date
Director of the York Center for Environmental Engineering and Science, NJIT

Dr. Somenath Mitra, Dissertation Co-advisor — Date
Professor of Chemistry and Environmental Science, NJIT

Dr. Robert Pfeffer, Committee Member — Date
Distinguished Professor of Chemical Engineering, NJIT

Dr. Joseph W. Bozzelli, Committee Member — Date
Distinguished Professor of Chemistry, NJIT

Dr. Rose Ann Dios, Committee Member — Date
Professor of Mathematics, NJIT

Dr. Trevor A. Tyson, Committee Member — Date
Assistant Professor of Physics, NJIT

BIOGRAPHICAL SKETCH

Author: Ling Yang

Degree: Doctor of Philosophy

Date: August 2004

Undergraduate and Graduate Education:

- Doctor of Philosophy in Environmental Science,
  New Jersey Institute of Technology, Newark, NJ, 2004
- Master of Science in Environmental Science,
  Nanjing University, Nanjing, Jiangsu, P.R. China, 1997
- Bachelor of Science in Environmental Science,
  Nanjing University, Nanjing, Jiangsu, P.R. China, 1994

Major: Environmental Science

Presentations and Publications:

Ling Yang and Daniel J. Watts, "The Influence of Surface Characteristics on Phytotoxicity of Nanometer-sized Alumina Particles", to be submitted to *Toxicology Letters*

To my mother and my husband
And in memory of my father

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CHAPTER 1

INTRODUCTION

1.1 Objective

The objectives of this dissertation are to determine the degree of toxicity, to investigate the underlying mechanisms through which the commercially available manufactured particulate materials impact on plant seedling root growth, as well as to identify those properties of nanoparticles that are important to their toxicity, using manufactured particles with nanometer sizes as models.

A testing system was established for determination of the toxicity and toxicological mechanisms of manufactured particles. Physical and chemical characterization techniques, coating techniques, along with toxicity tests were developed and applied simultaneously. An applicable phytotoxicity test was chosen from numerous toxicity test methods in the experimental portion of the study. The toxicity of particulate materials with different chemical compositions, including Al_2O_3, SiO_2, and TiO_2, was determined. The relationship between particle size, particle specific surface area, particle mass concentration, particle number concentration, or particle associate-chemical species and particle toxicity was evaluated in order to determine what particle properties are important to particle toxicity.

1.2 Background Information

Particle technology has experienced rapid development during the last 20 years. Particulate materials are now extensively used in industries (Roco, 1999). More recently, nanotechnology has brought manufactured nanoparticles into the work place as well as into the ambient atmosphere (Oberdorster, 2003). Epidemiological studies suggest that fine particles (particles with aerodynamic diameter smaller than 2.5 μm, PM2.5) have an association with various adverse human health effects including premature mortality, exacerbation of asthma and other respiratory-tract diseases, decreased lung function (Dockery and Pope, 1994, Schwartz et al., 1996, Pope, 1999, and 2000), and cardiovascular diseases (Dockery, 2001, Donaldson et al., 2001). These findings, coupled with the fast development of particle technology, have increased regulatory attention about the release of such particles into the environment and their potential impact on the health of individuals in the working area as well as public health. Studies are currently ongoing to investigate the environmental implications of particles with nanoscale sizes (Spurny, 1998). The published literature to date, however, focuses on human health effects of these particles. Few studies have taken into account that the specific properties of industrial type nanoparticles may play a vital role in the toxic effects of these particles.

A nanoparticle's interaction with a biological system is not just about the size of the very small particles. Compared to their siblings with sub-micrometer or micrometer sizes, they have significantly changed physical, chemical, and biological properties. Manufactured particulate materials at the same time can be transported by environmental media, such as water and air, and impose potential adverse effects on ecological systems. It is impossible to establish protection regulations or good manufacturing practices for the

particulate materials involved in industrial processes based on the very limited information available about the toxicity and environmental implications of artificial particles. The conclusion that the current regulations and standards can protect public health from any potential harmful effects of these particles remains questionable without enough information about the toxicity and toxicological mechanisms of the particles.

Simultaneous study of manufactured nanoparticles and the complexities in particle physical and chemical properties of airborne nanoparticles holds promise to clarify the health and environmental implications of the particulate materials. The administrator of the U.S. Environmental Protection Agency (EPA) issued new National Ambient Air Quality Standards (NAAQS) for $PM_{2.5}$ (airborne particles smaller than 2.5μm in diameter) in 1997, (USEPA, 1997), which is 65 mg/m^3 for 24-hr and 15 mg/m^3 for annual. The new issued $PM_{2.5}$ standard is based on extensive epidemiological evidence, which associates ambient particulate pollution with adverse health effects (Dockery and Pope III, 1994; Dockery, 1996 and 2001). Nevertheless, uncertainty and disagreement persist regarding which physical and chemical properties of particles influence health risks (NRC, 1998). The current standards for particle pollution are based on particles mass concentrations and particle sizes, and are the only standards of this type that are not chemical specific (NRC, 1998). Extensive studies have been performed in order to determine particle properties that induce the adverse health effects of particles indicated by the results from epidemiological studies, but no consistent results have been suggested (NRC, 1998). This controversy is largely due to the complexity and extreme diversity of the airborne particulate materials, which makes thorough and conclusive study impossible. The situation is even tougher for airborne nanoparticles because

airborne nanoparticles cannot be sampled with current sampling techniques, although it has been found that the urban particulate cloud may contain up to 10^5 nanoparticles per milliliter (Seaton et al., 1995).

Manufactured nanoparticles are well characterized and uniform in composition. The use of them as models reduces the complexity of the study of airborne nanoparticles, and makes the identification of the most toxic properties possible under more controlled conditions. Researches have been done on use of nanoparticles (e.g. TiO_2 particles of 21 nm (Churg et al., 1998), 29 nm (Renwick et al, 2001) and 50 nm (Stearns, et al., 2001), and carbon black particles of 14.3 nm (Renwick et al, 2001) as model systems of airborne particulate materials. The current research is however limited. First, it focuses on inhalation exposure, which is limiting because exposure to nanoparticles may occur via dermal contact or ingestion, in addition to inhalation. Second, the possible contribution to particle toxicity of chemical species that may be adsorbed on the particle surface has not been investigated. Study of the potential constituent is necessary because of the enormous surface area of nanoparticles that may result in the substantive surface adsorption of chemicals. In fact, studies on particles with larger sizes suggest that the surface chemistry of particles is related to their toxicity (Obot et al., 2002), and the association of particles and organic chemical species (e.g. polycyclic aromatic hydrocarbons) is more deleterious than either alone (Garçon et al., 2000, 2001a, and 2001b). Third, the current research cannot yet elucidate toxicological mechanisms of nanoparticles. It has been proposed in the literature that with similar mass in both cases, small particles are more toxic than particles with larger sizes because they have 1) smaller size, 2) larger specific surface area resulting in larger surface area with similar mass, and 3) larger numbers. Based on

this assumption alone, it is impossible to explain why ~ 800 nm SiO_2 particles are as toxic as ~ 20 nm TiO_2 particles (Oberdorster et al., 1994a). The toxicological mechanisms of particle – induced adverse health effects need further investigation. Fourth, published studies focus on human health effects of particles, with no contribution to the investigation of ecological implications of nanoparticles.

This dissertation will be on the front edge of studies on toxic effects of manufactured particles, and it is the first investigation of the effects of manmade nanoparticles on plant growth. The data on the most toxic particle properties collected in this study may provide a basis for additional studies, future regulations and pollution control strategies.

CHAPTER 2

LITERATURE REVIEW

A growing body of evidence has been provided by epidemiological studies that adverse health effects are closely associated with airborne particulate matter pollution. The evidence from the epidemiological studies has brought increasing interest to studies on airborne particles. Most of the important particle properties that may account for particle toxicities have been determined in recent years by studying fine and coarse airborne particulate materials. Toxicity studies have been performed to assess human health effects of the particulate materials. Toxicological mechanisms for particle-induced injuries have been proposed. The most recent fast development of particle technology and nanotechnology now requires studies on the health and environmental characteristics of manufactured nanoparticles. It has been found that particles with nanoscale sizes are more toxic than larger particles. Small sizes, large numbers of particles per unit mass, as well as large surface areas per unit mass, are the three major properties that have been suggested as being connected to the adverse human health effects of the ultrafine or nanoparticles.

2.1 Studies on Airborne Particulate Materials

Published studies to date have reported a number of the important particle properties that may account for particle toxicities. Substantial evidence that associates various adverse health effects with airborne particulate materials is provided by epidemiological studies.

Toxicity studies have been performed on human health effects of particulate materials. Toxicological mechanisms of particle-induced injuries have been proposed. All of these are discussed in the following sections.

2.1.1 Airborne Particle Properties

Table 2.1 lists the major airborne particle properties that have been considered in the literature so far (Spurny, 1998, Harrison and Yin, 2000, Zhang and Friedlander, 2000, NRC, 2001).

Table 2.1 Major Physical and Chemical Features of Airborne Particulate Materials

Physical Property	Chemical Property	
	Inorganic fraction	Organic fraction
Size	Metals (Fe, Al, Mn, Pb,	Polycyclic Aromatic
Surface area	Ca, Zn, Co, Cu, Si)	Hydrocarbons (PAHs)
Number based concentration (numbers per m^3 air)[a]	Ammonium	Alkanes, Alkenes
	Nitrate	Phenols
Mass based concentration ($\mu g/m^3$ air)[b]	Sulfate	Esters
-	Peroxides and free	Ketones
-	radicals	Carboxylic acids

[a] Numbers of particle per milliliter water is used as the unit for number based concentration in this study
[b] mg/ml is used as the unit for the mass based concentration in this study.

The most often-characterized particle properties are the physical properties (i.e., particle size, particle specific surface area, number based concentration, and mass based concentration), the inorganic fractions, PAHs, elemental carbon (EC), and organic carbon (OC). Organic components of particles including alkanes, alkenes, phenols, esters, ketones, and carboxylic acids are rarely determined, and are actually included in the analysis of the OC that also includes PAHs. The characterization techniques employed are similar in principle but instrumentally different. They may be referenced in many publications (Chow et al., 1996, Hughes et al., 1998, Morawska et al., 1998, Keywood et al., 1999, Zayed et al., 1999, Kim et al., 2000, Shi et al., 2000, Yatin et al., 2000, and Zimmermann et al., 2000). The reference to the literature of airborne particle chemical and physical characteristics facilitates the research on the manufactured particles, in which particle properties must be utilized to classify the particles into different categories.

2.1.2 Epidemiological Evidence of Airborne Particle-Induced Injuries

Short-term and long-term epidemiological studies have demonstrated the association between particulate matter pollution and various adverse health effects. In Europe, studies involving 21 cities and 5 years in the early-mid 1990s showed that "all-cause" daily mortality increased by 0.6% for each 10 $\mu g/m^3$ increase in PM10 (particulate matter with aerodynamic diameter smaller than 10 μm) (Katsouyanni et al., 2001). And in a study on hospital admissions, which involved 38 million people living in 8 European cities and lasting 3-9 years in the early to mid 1990s, it was demonstrated that hospital admissions for asthma and chronic obstructive pulmonary disease among old people were increased by 1.0% per 10 $\mu g/m^3$ PM10 (Atkinson et al., 2001). Moreover, admissions for

cardiovascular disease were increased by about 0.5% per 10 μg/m^3 total particulate materials and by about 1.1% per 10 μg/m^3 PM10 (Tertre et al., 2002). A series of long-term studies in Switzerland associated symptoms of bronchitis, but not asthma or allergy, in children in 10 different communities with PM10 at concentrations of 10-33 μg/m^3 (Braun-Fahrlander et al., 1997), which is well below the concentration level in many European countries. Studies in the US focusing on the 20 largest metropolitan areas, which were home to 50 million residents during 1987 to 1994, found that all–cause mortality increased by 0.5% per 10 μg/m^3 PM10, which is similar to the European results (Samet et al., 2000a, 2000b). Hospital admissions for chronic obstructive pulmonary disease in 10 cities with about 1.8 million elderly people increased by 1.5% per 10 μg/m^3 PM10, and for cardiovascular disease increased by 1.1% per 10 μg/m^3 PM10 (Zanobetti et al., 2000). In long-term studies, significant associations were reported between exposure to fine particles and bronchitis in children living in 24 US and Canadian communities (Dockery et al., 1996, Raizenne et al., 1996, Spengler et al., 1996).

2.2 Toxicity Studies on Human Health Effects of Particulate Materials

The toxicological mechanisms of particle-induced injuries are usually investigated and evaluated by laboratory *in vivo* and *in vitro* toxicity tests. According to the published studies, the toxicity tests are mostly applied to investigate human health effects of particles. The usual complement of tests include 1) mutagenicity assay, including Ames test, or human cell *in vitro* mutagenicity assay, 2) human cell *in vitro* immunology tests, including evaluation of immunoglobulin produced (IgE, IgG, IgA) from B-lymphocytes cell line as well as its gene transcription, cytokine protein releases and gene transcription

[interleukine (IL)-6, IL-8, and granulocyte-macrophage colony stimulating factor (GM-CSF), etc.] from cultured human airway epithelial cells *in vitro*, 3) human cell *in vitro* antioxidant activity tests, including tests for enzyme activities [superoxide dismutase (SOD) and glutathinone reductase (GR)] and glutathione status (reduced glutathione, GSH, and oxidized glutathione, GSSG) in human lung epithelial cells, and 4) *In vivo* inhalation toxicity studies using standard animal models. These are all discussed in more detail in the following Sections.

2.2.1 Mutagenicity Assay

2.2.1.1 Ames Test. The Ames test was introduced by Ames and Yamasaki in 1975, and revised in 1983 (Ames et al. 1975, Maron et al., 1983). This mutagenicity test is based on reversion of histidine-requiring auxotrophs to the wild type upon addition of mutagenic compounds. The most commonly used strains for the detection of particle mutagens are TA100 and TA 98 of *Salmonella typhimurium*. The mammalian microsome, S9, is used to metabolize indirect mutagens.

The Ames test requires that the chemical species associated with particles be extracted into organic solvents when applied in determining particle mutagenicity. The extract is then volume reduced and then solvent changed into, usually, dimethyl sulfoxide (DMSO), and added to the *Salmonella typhimurium* cultures. Pagono (1996) used the Ames test to investigate the mutagenic activity of total and particle-sized fractions. An association was reported between the particle diameter and the net revertants/mg: net revertants/mg increases with the decrease of the particle diameter. The chemicals tested were additionally analyzed by LC/GC-FID in other studies (van Houdt, 1990, Magusson et al., 2000) or GC/MS (Kado et al., 2000). The adsorbed organic chemical species

[usually polycyclic aromatic hydrocarbons (PAHs)] are the investigation targets in Ames tests of particle toxicity. The contributions of physical properties and the inorganic fraction (e.g. nickel) to the toxicity of the particles are not evaluated.

Another way of applying the Ames test is bioassay-directed chemical analysis. Bioassay-directed chemical analysis involves separation of a sample into fractions. Each fraction contains organic chemicals of similar functionality and polarity. The fractions are then tested in a bioassay to determine their toxicity. The bioassay results are used to direct attention to detailed chemical analysis of those fractions. By repeating this process of chemical separation and bioassay testing, the most mutagenic chemical species may be discriminated (Siak et al., 1985, Lwo, 1989).

2.2.1.2 Human Cell *in vitro* Mutagenicity Assay. Recently, a human cell mutagenicity assay procedure has been developed. Durant et al. (1998) tested the PAHs in airborne particles that could mutate human lymphoblasts. The cells used in the tests were h1A1v2 cells. Pedersen et al. (1999) applied the same cells and techniques in investigation of the mutagenicity of total organics adsorbed on respirable airborne particles. Further, Hannigan et al. (1998) used the human h1A1v2 cell mutagenicity assay for bioassay-directed chemical analysis of Los Angeles Airborne particulate matter, and PAHs were the investigation target.

2.2.1.3 Summary and Discussion of Mutagenicity Assay. The mutagenicity of particles is tested in mutagenicity assays. It has been confirmed that less than 80% of the chemicals that were found mutagenic in such screening assays are actually carcinogenic (Maron and Ames, 1983). Both the Ames test and the human cell mutation assay require the extraction of particles. Organic species that are adsorbed on the particles are extracted

into organic solvents. Inorganic species are left on the particles, uninvestigated. There is much evidence that heavy metals may be also mutagens, for example, Pb, and Ni (Scorecard, 2004). This classic toxicity test is thus targeted for organic chemical determination. Usually PAHs are found to account for most of the mutagenicity produced by extracted organics. The effects of other particle components are neglected. This problem may be solved if a new bioassay technique can be applied, in which the particles can be tested directly, with and without extraction of organic components, so that the effects of particles and inorganic species may be tested at the same time.

2.2.2 Human Cell *in vitro* Immunology Test

1) Summary of Immune System and Immune Response (Sell, 1987a, 1987 b)

The major constituents of the immune system are lymphocytes and macrophages. Lymphocytes may be divided into two categories: T-lymphocytes (T cells), and B-lymphocytes (B cells). A T cell is the precursor of a sensitized cell that is the basis for cell-mediated immunity, and may be subdivided into T helper cells, T cytotoxic cells, T suppressor cells, and other components. Immunoglobulin or antibody is secreted by plasma cells or activated B cells. A macrophage uptakes antigen nonspecifically and processes the antigen so that it may be recognized by a T cell or a B cell.

The immune response of the human body to an infection occurs first in the form of inflammation. Infected tissue cells release inflammatory cytokines and mediators such as interleukines (IL) and granulocyte macrophage colony stimulating factor (GM-CSF), which attract macrophages and lymphocytes to the infected site. Activated macrophages produce IL1. IL1 along with the antigen activates T cells. T helper cells, which are a subpopulation of T cells, are also activated and produce IL2. Other interleukines such as

IL3, IL4, IL8, IL10, etc., as well as T helper cells and IL2, activate B cells. B cells proliferate and differentiate into immunoglobulin-secreting plasma cells. B cells can also be activated directly by antigens.

There are five classes of immunoglobulin: IgA, IgD, IgG, IgM, and IgE. IgD and IgM are surface immunoglobulin of B cells, and are present under normal situations. While IgA, IgG, and IgE are secreted by plasma cells when antigen invades.

2) Human Cell *in vitro* Immunology Tests

Particles were suspended in culture medium in most published studies on particle toxicity in human cell *in vitro* tests. The suspensions were added directly into the cell cultures without additional sample preparations. This provides an excellent way to evaluate the toxicity of manufactured or manually coated particles.

The involvement of particles in the particle-mediated immune system response has been investigated intensively. Two categories of endpoints were evaluated. One is the level of cytokines and inflammatory mediators, in both protein production and gene expression. The other is the immunoglobulin protein production and gene expression. The testing system is based on human cell lines *in vitro*, including normal human airway epithelial cells (Rosenthal et al., 1994, Carter et al., 1997, Bayran et al., 1998, Quay et al., 1998, Boland et al., 1999, Takizawa et al., 2000), virus transformed human bronchial epithelial cell line (Abe et al., 2000), and human Epstein-Barr virus transformed isotype switched IgE producing B cell line (Takenaka et al., 1995, Tsien et al., 1997). All of these studies reported that particles could enhance the production of cytokines and immunoglobulin, namely, interlukine8 (IL-8) and immunoglobulin E (IgE). Takenaka et al. (1995), Boland et al. (1999), and Bayran et al. (1998) studied the involvement of

PAHs extracted from the particles on cytokine or IgE production. Tsien et al. (1997) and Saxon et al. (2000) evaluated the effect of phenanthrene, and suggested that phenanthrene could significantly enhance the IgE protein production. Kawasaki et al. (2001) reported that benzene-extracted particle components showed effects mimicking the effect of airborne particles on IL-8 protein production and gene expression.

The protein level of cytokines and immunoglobulin was determined by commercially available ELISA (Enzyme Linked Immuno-sorbent Assay) kits. The determination of gene expression of cytokines or immunoglobulin was performed by the technique of RT-PCR (reverse transcription/polymerase chain reaction).

The particles evaluated in these studies were mainly diesel exhaust particles (DEPs) with a few exceptions of manufactured particles, such as TiO_2 and Fe_2O_3 studied by Stringer et al. (1996). The size of the manufactured particles however was simplex (diameter of 1 μm). No studies have been reported to date on the application of immunology tests on evaluation of adverse health effects produced by nanoparticles.

2.2.3 Human Cell *in vitro* Antioxidant System Response Test

Particles are suspended in culture medium in the antioxidant system response test, and added to cell cultures without any extraction or preparation steps. This offers a good way to investigate directly the toxicity and health effects that may be particle induced by the particle physical and chemical properties.

Oxidant stress imposed by particles is suggested to be central to the determination of the particle's pathogenicity. Ferin et al. (1992) found that acute pulmonary inflammation was detectable following inhalation of ultrafine (20 nm in diameter) TiO_2 but not after inhalation of normal-sized (200 nm in diameter) TiO_2. Donaldson et al.

(1996) used a sensitive DNA assay and investigated the free radical activities at the surface of the ultrafine (500 nm) and nanometer (20 nm) TiO_2, and found that nanometer TiO_2 was much more active than ultrafine TiO_2. They further evaluated the DNA damage by adding a known free hydroxyl radical scavenger, mannitol, to the cell cultures that were being treated with the particles, and found that after adding mannitol, the DNA damage was ameliorated, showing that the hydroxyl radicals were involved. They suggested that the free radical activity might be a factor that influences the redox balance within the cells by depletion of glutathione (GSH), and the redox balance was an important factor in the induction of transcription of immunologically-related genes and genes that must be activated for proliferation to occur in cells.

Kadiiska et al. (1997) demonstrated the free radical production *in vivo* by introducing oil fly ash (1.95 ± 0.18 μm) into the lungs of rats. They found that the generation of free radicals appeared to be associated with soluble metals. Carter et al. (1997) investigated the involvement of metals in oil fly ash-mediated cytokine production. Oil fly ash particles contain the metals vanadium, nickel, and iron. Normal human bronchial epithelial cells produced significant amounts of cytokines including IL-8 and IL-6 when exposed to oil fly ash particles, but the effects were obviously inhibited by either deferoxamine (a metal chelator) or dimethylthiourea (a free radical scavenger), indicating that the metals and the free radicals play an important role in the enhanced production of IL-8 and IL-6.

Stringer and Kobzik (Stringer and Kobzik, 1998) hypothesized that oxidant mechanisms might be involved in the cellular response to particles. They added the antioxidant N-acetylcysteine (NAC) in the testing system and found that NAC could

decrease the particle-induced IL-8 production. Casillas et al. (1999) stated that diesel exhaust particles might generate reactive oxygen species that could be abrogated by antioxidants. Dellinger et al. (2001) examined samples of $PM_{2.5}$, and found large quantities of radicals with characteristics similar to semiquinone radicals, which are known to undergo redox cycling and ultimately produce biologically damaging hydroxyl radicals. They also tested the damage induced by $PM_{2.5}$ samples to DNA in human cells. The damage could be abolished by an antioxidant including SOD, catalase, and deferoxamine.

It can be concluded from these studies that oxidant stress may play a pivotal part in particle-mediated human organism injury. Morin et al. (1999) tested the antioxidant system response in rats' lung slice cultures. They determined glutathione (GSH) level and superoxide dismutase (SOD) activity with or without particle exposure. GSH level was markedly decreased after 1-h exposure of diesel exhaust particles. And SOD activity was also decreased. The authors suggested that the oxidant stress, for example, the production of oxygen radicals such as $\bullet O_2$ and $\bullet OH$, could explain the decrease of GSH level and SOD activity.

Garçon and his co-workers (Garçon et al., 2000, 2001a, 2001b) applied the techniques of antioxidant system determination to evaluate the toxicity of 1 – μm Fe_2O_3 particles (pure and coated) in human lung cells A549. They evaluated the activities of SOD, glutathione peroxidase (GPx), glutathione reductase (GR), and the status of glutathione: reduced form (GSH) and oxidized form (GSSG). The increases in GSSG/GSH, in SOD activities, as well as in the GR activities were associated with the induction by the particles.

Considering the fact that various particles (TiO_2, oil fly ash, diesel exhaust particle, PM10, and Fe_2O_3 among others) can impose oxidant stress on the cells, and that the antioxidant system in normal human cell cultures reacts to the oxidant stress, and that the evaluations of SOD and GR activities as well as of the GSSG/GSH are convenient to perform, the antioxidant system response test becomes one candidate toxicity test for a particle toxicity testing system.

2.2.4 *In vivo* Inhalation Toxicity Test

An *in vivo* inhalation test is widely used to investigate the toxicity and health effect of particles. Donaldson et al. (1996) used rats to study the inflammogenic effects of micrometer-sized and nanometer-sized particles. They found that material that is relatively inert in the form of micrometer-sized particles could be highly inflammogenic for particles in the nanometer size range. The effect of inhalation, deposition in the respiratory system, phagocytosis by macrophages, and clearance of particles was investigated by studies using dogs (Clarke et al., 1966, Kalmykova et al., 1980, Wolff et al., 1985), donkeys (Berger et al., 1978), rats (Bellmann et al., 1983, Reichrtova et al., 1986, Oghiso et al., 1986, Tanaka et al., 1986, Brightwell et al., 1986), rabbits (Brain et al., 1984), guinea pigs (Conner et al., 1988) and hamsters (Geiser et al., 1994), etc. *In vivo* evidence of free radical formation after exposure to air pollution particles was demonstrated using rats (Maria et al., 1997). Recently, the effect of inhaled ultrafine particles on the allergic airway response was studied using dogs (Barret et al., 2003). Sometimes, human volunteers help to study the effect of particles on the human respiratory system by inhaling particles (Curry et al., 1975).

Chamber exposure to an aerosol containing particles is usually involved in the *in vivo* test. The *in vivo* test is helpful in the investigation of particle transportation and clearance in the respiratory system, but it is not applicable for the initial study of the toxicological mechanism of inhaled particles, because the particles can affect various targets in the respiratory system (e.g. mucous cilia, macrophages, and alveolar epithelium cells, among others) and cause many adverse effects (e.g. alveolar macrophage phagocytic and enzymic activities, slow clearance, and inflammation, among others). It is difficult to distinguish either the particle that is affecting targets or what kind of adverse effect the particle is inducing, because the whole respiratory system of the test animal is involved. The *in vivo* test is good as the final investigatory step of the health effects of particles though.

2.2.5 Summary and Discussion of Studies on Human Health Effects of Particles

Human health effects of particles were determined by mutagenicity tests, *in vivo* tests, and *in vitro* tests including immunology tests and antioxidant system response tests in the literature. Mutagenicity tests are limited in clarification of effects and toxicological mechanisms of particulate materials because only the organic components of the particles (usually PAHs) are investigated in mutagenicity tests. Studies using the latter three tests have yielded consistent results, i.e., increase of IL-8 and IgE production, and increase of SOD and GR activities as well as of GSSG/GSH ratios. Studies of human health effect of particles that have been published so far focus on the reactions of the microorganisms , human cells *in vitro*, or animals *in vivo*, which is necessary but not enough to elucidate the toxicological mechanisms of particles. Particle properties should be considered to get thoroughly understanding of the toxicological mechanisms of particles.

2.3 Phytotoxicity Studies using Plant Systems

Toxicity of particles on plant systems is rarely investigated up to date. Lifecycle human health protection however means protection of the global environment as a whole. This means studies on ecological implications of pollutants are as important as studies directly using human body systems. A testing method must be decided in order to study the effect of particles on plant systems.

Plant system tests such as the micronucleus test in *Vicia faba* root tips and the *Tradescantia* micronucleus bioassay have been used for environmental mutagen screening (Ma, 1981, 1982, Degrassi and Rizzoni, 1982, Marco, 1990, Cotelle, 1999). Recently it has been found that air pollution particles are available to and cause genotoxic damage to plants. (Poma et al., 2002). The published phytotoxicity studies observed a common phenomenon that the root elongation of the plant was inhibited significantly if the studying subjects are mutagenic or toxic.

Seed germination and root elongation toxicity tests are recommended by the U.S. EPA as ecological effects tests of pesticides and toxic substances (EPA, 1996). This approach was validated as an indicator to assess the phytotoxicity of halogen-substituted phenols and anilines (Wang et al., 2001). More recently, the relation between aluminum toxicity and oxidative stress was studied for *Zea mays* by determining the root elongation impacts as well as the peroxidase, catalase, and superoxide dismutase activities in root tips (Boscolo et al., 2003).

2.4 Coating Techniques

Studies of the model particulate system by coating test chemicals onto airborne particles date back to the 1980s. Bond et al. (1984) investigated the dog pulmonary macrophage metabolism of free and particle-associated benzo[a]pyrene (BaP). The free form was a BaP solution, and the particle-associated form was BaP vapor coated onto diesel exhaust particles. Bjorseth et al. (1985) compared the elution rates of BaP-coated coal fly ash particles that had been prepared by either vapor coating or liquid coating. Mumford et al. (1986) evaluated the bioavailability of 1-nitropyrene vapor coated coal fly ash particles and studied their uptake by alveolar macrophages. Ball et al. (1986) studied the metabolism, disposition and molecular binding of 1-nitropyrene vapor-coated diesel particles. Lee et al. (1989) established a generator for aerosols that had diesel particulate cores (freshly generated diesel particles) and coatings of polycyclic aromatic compounds to study the dinitropyrene formation in diesel particles.

Studies on an airborne particle model system prepared by coating chemicals onto pure particles were not reported until recently. The investigations of health effects produced by this type of model system were reported even later. Cheu et al. (1997) studied the uptake, metabolism and DNA binding of BaP vapor coated Fe_2O_3 and Al_2O_3 particles. No health effect tests, as far as what is known, had been performed until that time. Starting in the year of 2000, Garçon et al. (2000, 2001a, 2001b) performed a series of investigations on antioxidant system disruption by polycyclic aromatic hydrocarbons coated hematite particles in human lung cells *in vitro* or human lung epithelial cells *in vitro* or lung tissue cultures *in vivo*. They coated benzo[a]pyrene and/or pyrene onto Fe_2O_3 particles of 1 μm in diameter by liquid phase coating, in which benzo[a]pyrene

and/or pyrene was dissolved into chloroform and added to the particles. The results from their study demonstrated that the enzyme activities, including the activities of superoxide dismutase and glutathione reductase, increased when the cells were exposed to the particles, whereas the glutathion status, i.e. the ratio of reduced glutathion to oxidized glutathion, decreased, indicating the presence of oxidative stress in the cells.

Another coating technique that was applied to the study of biological effects of particles was reported by Jang et al. (1999). In their study, a high-pressure aerosol generator and outdoor Teflon film chambers were used to investigate the adsorption of semi volatile organic compounds (SOCs) on fine inorganic road dusts. This study established and evaluated a predictive mathematical model for gas-particle partitioning of SOCs on fine atmospheric inorganic dust particles.

2.5 Studies on Manufactured Nanoparticles

Conner et al. (1988) investigated lung injury in guinea pigs *in vivo* caused by inhalation of 50 nm zinc oxide (ZnO), and found that exposure to ZnO at 5.9 or 12.1 mg/m^3 resulted in the pulmonary damage in the animals. More recently, preliminary toxicity studies on nanoparticles, presented at the 2003 American Chemical Society (ACS) New Orleans meeting, suggest that inhaled 20-nm diameter but not 130-nm particles made from polytetrafluoroethylene (PTFE, Teflon) had adverse effects on the respiratory system in rats (Oberdorster, 2003). This result is consistent with previous studies, in which 16 nm PTFE, but not particles larger than 100 nm, was found to cause severe acute lung injury to rats when inhaled for only 15 min at 50 $\mu g/m^3$ (Johnston et al., 2000). Similarly, it has been demonstrated that TiO_2, Al_2O_3, and carbon black particles with diameters smaller

than 100 nm have exceptional toxicity and are more likely to induce the development of particle-mediated lung diseases than the same mass of larger particles (Donaldson et al., 1998, and 1999). Compared to larger particles, nanoparticles have high deposition efficiency in the lower respiratory tract and slow clearance rates (Spurny, 1998, Oberdorster et al., 1994b). Churg (Churg et al., 1998) investigated the uptake of 120 nm and 21 nm TiO_2 particles in a tracheal explant system. Compared to 120 nm particles, 21 nm particles appeared to enter the epithelium faster (3 days vs. 7 days), and a greater proportion of them was translocated to the subepithelial space due to the larger number of particles applied with the same mass (epithelial to subepithelial volume ~2:1 for 21 nm particles vs. ~1:1 for 120 nm particles). More recently, Renwick et al. (2001) demonstrated that nanometer sized TiO_2 and carbon black particles impair macrophage phagocytosis at a lower dose than submicron sized particles (0.39 vs. 0.78 μg per m^2 of alveoli). Other studies have reported that when inhaled as single particles, particles smaller than 50 nm can be fatal toxic (Oberdorster, 1996).

It is suggested in the literature that the ultrafine or nanometer scaled particles are more toxic than larger particles because they have a larger surface area per unit mass, which means they can adsorb more toxic chemicals on their surface than can the same mass concentrations of the coarser particles. Penn et al. (1999) studied the particle size dependence of organic adsorption, and found that the maximum adsorption per unit surface area was higher for smaller particles. The large surface area per unit mass of ultrafine- or nano-particles would allow increased interaction between particles and epithelium cells (Donaldson et al., 1998), and could result in greater toxicity of the particles (Oberdorster et al., 1992 and 2000).

It is also suggested in the literature that the ultrafine or nanometer scaled particles may be more toxic than larger particles because they have smaller sizes and therefore they can penetrate deeper into the respiratory system (Pui, 1997, Churg et al., 1998) and may remain in the bronchioles and alveoli for months (Holt, 1987).

They may overload the particle clearance system, especially the macrophages that are essential to the clearance of fine and ultrafine or nanometer scaled particles. The overload state results from the large numbers per unit mass of ultrafine- or nano-particles. If the particles are intrinsically toxic, they may injure or kill the macrophages in addition to overloading the system. Either overload or injury leads to failure in clearance of the particles due to the inactivity of the macrophages. The inhaled particles can remain in the bronchioles and interact with epithelial cells, resulting in allergic inflammation, injury to the antioxidant system, and/or cancer (Spurny, 1998).

Sizes, numbers per unit mass, surface area per unit mass, and perhaps intrinsic toxicities are the particles' properties that have been suggested to be responsible for the toxic effects of fine and coarse particles. Considering that properties such as the surface characteristics of ultrafine- or nano-particles are much different than those for the fine and coarse particles (Roco, 1999), the toxicological mechanism of nanoparticles needs further investigation.

CHAPTER 3

RESEARCH SUMMARY AND METHODOLOGY

Current research on toxic effects and the mechanisms of artificial particulate materials as well as airborne particulate materials was reviewed in Chapter 2. Human health effects and the mechanisms associated with these health effects have been widely studied. Ecological implications of the particles are typically not of concern in the published studies. The effects of particles on plant growth were studied in this dissertation using the EPA recommended root elongation test method with slight modification. Classic particle physical and chemical characterization techniques including Fourier Transform Infrared (FTIR) spectroscopy, Scanning Electron Microscopy / Energy Dispersive Spectrometry (SEM/EDS), and Brunauer–Emmett–Teller (BET) algorithm, among others, were applied. Statistical analysis was performed using the procedures of *one-way Analysis of Variance*, or *one-way ANOVA*, as well as the *Student's t-test*.

3.1. Research Summary

This study focus on the following two areas:

1) Toxicity evaluation of manufactured nanoparticles.
2) Investigation of particle properties that contribute to particle toxicity.

Commercially available manufactured particulate materials with different chemical compositions including aluminum oxide, silicon dioxide, and titanium dioxide were purchased for the study. Particle aggregate sizes and specific surface areas were

characterized. The effects of these particles on plant seedling root growth were investigated. The toxicity threshold values, IC_{10} or EC_{10} (concentration that produces an 10% inhibitory or enhancement effect), were determined.

Particles with two to three size scales and with two to three specific surface areas were obtained in order to investigate the particle properties that contribute to particle toxicity. The effects of these particles on plant seedling root growth were compared according to the particle sizes as well as the particle specific surface areas. Phenanthrene (Phen), an important member in the family of polycyclic aromatic hydrocarbons (PAHs), was loaded on the particles. The effects of Phen-loaded particles on seedling root growth were investigated to evaluate the influence of Phen on particle toxicity.

Mechanisms of how the particles influence seedling root growth were investigated by the techniques of FTIR and SEM/EDS.

3.2 Research Methodology

3.2.1 Physical and Chemical Characterization

3.2.1.1 Analysis for the Sizes of the Aggregates of the Particles. Particles exist in water suspensions as aggregation forms, not individual particles. The sizes of the aggregates of the manufactured particles were determined by particle size analysis instrumentation, using a Coulter LS-230 for coarse and fine particles (particles smaller than 10 μm and larger than 1μm), and a Coulter N4+ for ultrafine particles (i.e., particles smaller than 1μm).

Particles were suspended in Milli-Q water and sonicated for three hours when using the Coulter N4+ for the size determination. This three-hour sonication duration was

determined in a preliminary study by measuring the size of the 14-nm alumina particles after a series of sonication periods: one hour, two hours, three hours, and four hours. A 3 ml sample of the suspension of the 14-nm particles at the concentration of 2 mg/ml (20 mg particles to 10 ml Milli-Q water in this study) was pipetted to a cuvet after each sonication duration, and analyzed by the Coulter N4+ three times (i.e., three continuous runs using the same sample). The particle sizes determined from the three runs were averaged to get a "run average" value, as well as a standard deviation. The four "run average" values were compared. It was found that after three hours of sonication, the particle size did not change dramatically. The three hours of sonication was thus determined to be sufficient to obtain non-reducing particle sizes, and was applied for either particle size determinations or phytotoxicity studies.

The particles that were size-analyzed by the Coulter N4+ include the 13-nm (individual particle size according to the manufacturer) alumina particles, the 14-nm (individual particle size according to the manufacturer) silica particles, the 21-nm (individual particle size according to the manufacturer) titania particles, the nano-sized silica particles (determined by this procedure to be 161.2 nm), the submicron silica particles (determined by this procedure to be 667.6 nm), and the submicron titania particles (determined by this procedure to be 0.96-μm) (the sizes of the aggregates of these particles as determined by the Coulter N4+ is given in Table 3.2).

The concentration of 2 mg/ml (20 mg to 10 ml in this study) was used for the size determination for the nanoparticle aggregates including the 13-nm alumina particles, the 14-nm silica particles, the 21-nm titania particles, and the 161.2-nm silica particles. The concentration of 20 mg/ml (200 mg to 10 ml in this study) was used for the size

determination of the submicron silica particles and the submicron titania particles. The volume of 3 ml of each particle suspension was transferred to a cuvet for analysis.

The size analysis performed by the Coulter LS-230 followed a different procedure. The Coulter LS-230 was used to determine the aggregate size of particles with micron sizes, i.e., the micron alumina particles in this study, which was determined as 1.00 μm. The micron alumina particles were soaked in Milli-Q water at 20 mg/ml (200 mg particles to 10 ml Milli-Q water in this study). The particle suspension was pipetted into the sample inlet of the instrument, which was filled with one liter of Milli-Q water before adding the particle suspension. The particle suspension was pipetted in until the obscuration approached to 8%, which is a requirement of the normal operation of the instrument. Normally, only 5 to 10 drops of the 20-mg/ml particle suspension were added to obtain the obscuration of 8%. The concentration of the particle suspensions that was being analyzed was about 0.007 mg/ml. A high pump speed (50%) was used. Internal sonication was functioning continuously during the measurement. The high pump speed and the internal sonication were used to prevent aggregation of the micron alumina particles. Particle size was reported as the mean value and standard deviation of the particle diameter (see Table 3.2).

In the following text of this dissertation, the particle sizes that are used to address the 1.00-μm alumina, 0.96-μm titania, 667.6-nm silica, and 161.2-nm silica are sizes of the particle aggregates. The sizes of the individual particles that are reported by the manufacturers are used for addressing the other particles including 13-nm alumina, 14-nm silica, and 21-nm titania.

3.2.1.2 Particle Surface Area Analysis. The specific surface area of the manmade particles that were studied in this dissertation was determined by an instrument using a mathematical operation based on Brunauer–Emmett–Teller (BET) theory, which is the calculation of the particle surface area based on the correlation between the total particle surface area and the amount of adsorbed nitrogen. The specific surface area of the 14-nm silica particles was obtained from the material datasheet supplied by the manufacturer.

The determination of specific surface area of the particles followed the standard procedure that is given in the manual of the instrument, which is a Nova® *e*-3200 device from Quantachrome Instruments. Dry and calibrated glass tubes with a calibrated glass rod inserted in each tube were weighed before adding the particles used in this study. The particles were added into the glass tubes, which were then connected to the vacuum ports of the instrument with the glass rods inserted, and degassed at 250°C for three to four hours. The glass tubes, along with the particles that were contained and the glass rods that were inserted, were left under vacuum at the end of the degassing until the temperature of 250°C lowered to the room temperature (which takes about 3 hrs). The glass tubes that contained particles as well as the glass rods were then weighed again. The weight of the particles was obtained by difference. The weights of the particles as determined by this way were given in Table 3.1.

Table 3.1 The Amount of the Particles (grams) used in the Determination of the Particle Specific Surface Areas (m^2/g)

Particle	1.00-μm alumina	0.96-μm titania	667.6-nm silica	13-nm alumina	14-nm silica	21-nm titania	161.2-nm silica
Amount (g)	1.9161	1.3084	0.1482	0.0211	0.0244	0.0553	0.0323

The glass tubes, which contained the particles as well as the glass rods, were connected to the analysis ports of the instrument, and then inserted into liquid nitrogen. The particle specific surface areas were analyzed under nitrogen gas streams and calculated by the instrument according to Brunauer-Emmett-Teller (BET) theory. The BET points in the analyses were five.

3.2.1.3 Determination for Particle Surface-Associated Chemical Species. The investigation of particle surface associated chemical species as well as particle surface characteristics was done by FTIR. 10 mg of particles or chemical compounds (phenanthrene in this study) were mixed in 200 mg of ground KBr (IR grade, 99+ %, Fisher Scientific). The powder was then pressed into a pellet (Ø = 10 mm). Each pellet contains 3 ~ 5 mg of particle. 2500 scans were performed on each pellet. The background spectrum was also 2500 scans that were performed on a blank KBr pellet. A Mattson Research Series FT-IR was used for the purpose of this study. The FTIR spectra were used 1) to determine whether or not phenanthrene had been loaded on the particles, and 2) to investigate the possible interaction between the particle-surface-adsorbed phenanthrene and the particle surface.

3.2.1.4 Electron Microscopy Analysis. Scanning Electron Microscopy (SEM) was used to investigate the distribution status of the particles in plant root systems. Particle element composition was analyzed by an energy dispersive spectrometry (EDS) under 5 keV. EDS spectra were collected for 20 minutes. The details of the procedures for preparing specimens and SEM/EDS analysis are given in Section 3.2.4.

3.2.2 Coating Technique

The coating technique candidates include vapor coating, liquid phase coating, and supercritical carbon dioxide coating. The liquid phase coating technique was chosen in this study, because liquid phase coating:

1) is convenient and easy to perform,
2) provides easy management of the load amount of the chemicals, which is the most important result wanted in this study, and
3) provides reproducible even coating of the particles.

A certain amount of phenanthrene (Phen) was dissolved in 4 ml of acetone in liquid phase coating. The amount of Phen was determined according to the mass (g) of particles, the specific surface area (m^2/g), the molecular cross section area ($Å^2$), and the molecular weight (g/mol) of Phen. The equation used for the calculation and the details of the amount of Phen and particles are given in the respective sections (see Chapter 5).

Particles were weighed. Nanoparticles (i.e., 13-nm alumina, 14-nm silica, and 21-nm titania) were dispersed in the 4-ml phenanthrene-acetone solution (see Table 5.1 on page 85 for the amount of phenanthrene in each solution) by continuous stirring by a glass rod by hand during the dispersion process. Particles including the 161.2-nm silica, 667.6-nm silica, 0.96-μm titania, and 1.00-μm alumina were dispersed into the 4-nm phenanthrene-acetone solution by continuous stirring using a glass rod by hand, and the suspensions were sonicated for 1 hr at room temperature. All of the particles studied in this dissertation were also dispersed in 4 ml of acetone to make non-phenanthrene-loaded (nonloaded) particles (blank samples) following the same dispersion procedures. The slurries were then left at 38 ± 1°C under vacuum overnight and kept in vacuum for

another 24 hrs to ensure the removal of the residue acetone (Garçon et al., 2000 and 2001). The Phen-loaded particles, as well as the nonloaded blank acetone treated particles were transferred to small vials, sealed, and stored in the dark at room temperature.

3.2.3 Phytotoxicity Test

Six plant species, *Z. mays* (corn), *C. sativus* (cucumber), *A. sativa* (oat), *G. max* (soybean), *B. oleracea* (cabbage), and *D. carota* (carrot) were used in the phytotoxicity tests. The exact plant species that were used for testing of each kind of particles, as well as of phenanthrene, are given in the according sections of Chapter 4 and Chapter 5.

Seeds of these six plant species were cultured into seedlings before exposure to the particles. 200 ~ 400 seeds for each test batch were soaked in 200-ml 10 % sodium hypochlorite solution for 10 minutes (EPA, 1996) to assure sterility. They were rinsed three times, and then soaked overnight in 200-ml Milli-Q water in an incubator at 25 ± 1°C except for *C. sativus* seeds. *C. sativus* seeds were soaked in 200-ml Milli-Q water in an incubator at 25 ± 1 °C for 1 hr after being treated with sodium hypochlorite and rinsed three times, and then geminated.

The seeds of the other five plant species were transferred in the next morning, uniformly to filter papers placed in 100 × 15 mm sterile disposable Petri dishes. Each dish contained 5 ml of Milli-Q water. Seeds were separated from each other by about 1 cm in every dish, and allowed to germinate at 25 ± 1 °C in the dark for 24 hrs to 72 hrs (depending on the species) prior to particle exposure, until the primary root of most seedlings elongated to about 20 ~ 30 mm. The time for culturing seeds into seedlings that with 20 ~ 30 mm of primary root length was 72 hrs for *Z. mays*, *A. sativa*, and *G. max*. 48 hrs were required for the seeds of *D. carota* and *B. oleracea* to germinate to seedlings

with 20 ~ 30 mm of primary root length. Seeds of *C. sativus* took 24 hrs to germinate into seedlings with 20 ~ 30 mm of primary root length.

Milli-Q water was renewed every 24 hrs during the germination process.

Seedling exposure to the particles seedlings was done in a particle–Milli-Q water suspension. Particles were weighed and suspended in Milli-Q water by sonicating for three hours to make a series of concentrations: for 20 mg/ml particle suspensions, 400 ± 0.15 mg of particles were added into 20 ml Milli-Q water; for 2 mg/ml particle suspensions, 40 mg ± 0.15 mg of particles were added into 20 ml Milli-Q water; for 200 μg/ml, 40 ± 0.15 mg of particles were added into 200 ml Milli-Q water; and for 20 μg/ml, 4 ± 0.05 mg of particles was added into 200 ml Milli-Q water. The balance used for weighing the particles has the accuracy of 0.01 mg. The particles tested in this study include original particles (which are the particles without any treatment), nonloaded particles (which are the particles treated with 4 ml of acetone but without phenanthrene loading), and phenanthrene-loaded particles. The pH of the particle suspensions was measured before being dispensed into Petri dishes. The particle suspensions were then dispersed into Petri dishes, with 5 ml for one dish, and altogether 15 ml for one concentration. The Petri dishes contained one filter paper in each dish before the dispersion of the particle suspensions. The seedlings were exposed to a concentration series and to a water only control (negative control). The negative control, i.e. the blank, was 5 ml of Milli-Q water. Three replicates were tested for each concentration and control. The three replicates contained 10 seedlings per dish and altogether 30 seedlings for each concentration.

Seedlings were transferred to the filter paper in the dish, which contained 5 ml of Milli-Q water or particle suspension and had been marked at the outside bottom of the dish for the purpose of identification and numbering of each seedling. The seedlings in each dish were separated from each other by about 1 cm. The exposure process was done at 25 ± 1°C in the dark for 24 to 72 hrs. The pH of the particle suspensions in the Petri dishes was determined by pH test strips. The length of the primary root or shoot of each normal seedling was measured before and after exposure. Seedlings with broken roots were excluded in the results.

Root elongation (RE) during the exposure period was calculated using the equation (1). A unified method of data analysis must be used for comparative purposes, because the root elongations of seedlings are not constant among different test batches and different plant species. A Relative Root Growth (RRG) was calculated for this purpose, based on what was proposed by Schildknecht (Schildknecht and Vidal, 2002), using the equation (2).

$$RE\ (mm) = L_{after}\ (mm) - L_{before}\ (mm) \tag{3.1}$$

$$RRG = RE_{sample}/RE_{control} \tag{3.2}$$

where L_{after} and L_{before} refer to the measured root lengths after and before exposure, respectively.

The phytotoxicity of phenanthrene (Phen) (98%+, assay, Fisher Scientific) was tested. 0.8400 grams of Phen was dissolved in 10 ml acetone (HPLC, Fisher Scientific) resulting the concentration of 84.00 mg/ml. A small volume of this solution (100 µl) was

added to 30 ml Milli-Q water to make a concentration of 0.28 mg/ml, which was the highest amount of Phen that has been loaded onto the particles. The mixture was left overnight under the hood at room temperature. The mixture was then dispensed into three Petri dishes, with 5 ml to each dish, and then tested.

Another phytotoxicity evaluation of phenanthrene was done at concentration of 0.02 mg/ml. 0.0603 grams of Phen was dissolved into 10 ml acetone to make the concentration of 6.03 mg/ml. 100 μl of the solution was added to 30 ml of Milli-Q water to get the concentration of 0.02 mg/ml. The mixture was left overnight under the hood at room temperature, and then dispensed into three Petri dishes with 5 ml to each dish to get tested.

The blank for the phytotoxicity evaluation of phenanthrene was 30 ml Milli-Q water plus the equivalent volume (100 μl) of acetone, which was also left overnight in a vacuum together with the particles that were being loaded with Phen.

3.2.4 SEM Study of Particle–Affected Root Samples

The lengths of the primary roots of the seedlings were measured after exposure. They were then cut into 5 mm (by length) sections. Fixation was done by soaking the root pieces in 3 ml Karnovsky's fixative (Paraformaldehyde-Glutaraldehyde solution containing 4.00% formaldehyde and 6.25% glutaraldehyde, from Electron Microscopy Sciences) at 2~4°C for 3 hrs. The samples were rinsed three times with 0.1 M phosphate–buffered saline (PBS) after fixation at room temperature, with 20 min and 3 ml of 0.1 M PBS for each rinse. The root tissues were post-fixed with 3 ml 2% osmium tetroxide overnight at room temperature, and dehydrated with 3 ml graded ethanol at room temperature: 30% ethanol for 15 min, 50% ethanol for 15 min, 70% ethanol for 15 min,

90% ethanol for 15 min, 95% ethanol for 15 min, and three changes of 100% ethanol for 15 min each. The samples were then dried with series of hexamethyldisilazane (HMDS) solutions as follows: 100% ethyl alcohol/HDMS (2:1) for 15 min at room temperature, 100% ethyl alcohol/HDMS (1:2) for 15 min at room temperature, and two changes of 100% HDMS for 15 min each at room temperature. The HMDS solutions used were also 3 ml for each concentration. The specimens were left to air–dry overnight, mounted, and sputter coated with gold for SEM observation. The SEM used is a LEO 1530 VP FESEM/EDS system (Leo Electron Microscopy). An accelerating voltage of 5 keV was used. The magnification is 5kX. The EDS spectra were collected for 20 minutes for each specimen.

3.2.5 Statistical Analysis

The results were expressed as mean ± standard deviation (SD), along with 95% confidence interval The statistical analysis was performed using the *Student's t-test*, as well as the *one-way ANOVA procedure*.

3.2.5.1 ***Student's t-Test.*** The *Student's t-test* is available as a program through an Internet site (physics.csbsju.edu, 2004). Statistically significant difference is reported when the probability of the result assuming the null hypothesis (p) is less than 0.05. At this point, the calculated t value is larger than the upper critical t value in the *Student's t distribution table* with the same degree of freedom and significance level of $\alpha = 0.05$. The results from the triplicate samples were compared first. If one group of data is significantly different from the other two groups, this group of data was discarded.

3.2.5.2 ***One-way ANOVA.*** The *ANOVA procedure* is one of the most powerful statistical techniques, which can be used to test the hypothesis that the means among two

or more groups are equal. Since in this study, only one factor, concentration, is concerned. The *one-way ANOVA procedure* was used. The null hypothesis of *one-way ANOVA* is that there is no difference in the population means of the different levels of factor A (the only factor). The goal of this procedure is to split the total variation in the data into a portion due to random error and portions due to changes in the values of the independent variable(s). The variance of total measurements in the data can be given as:

$$s^2 = \frac{\sum_{i=1}^{n}(y_i - \overline{y})^2}{n-1} \tag{3.3}$$

where $\overline{y}$ is the mean of the total measurements, n is the number of measurements, and y_i is the value of each measurement. The numerator in Equation 3.3 is called the *sum of squares* of deviations from the mean (Total SS), and in *one-way ANOVA is* split into two components, *sum of squares of treatments*, SST, and *sum of squares of error*, SSE:

$$SST = \sum_{i=1}^{k} n_i(\overline{y}_i - \overline{y})^2 \tag{3.4}$$

$$SSE = \sum_{i=1}^{k}\sum_{j=1}^{n_i}(y_{ij} - \overline{y}_i)^2 \tag{3.5}$$

where k is the number of groups, n_i is the number of values in the group, $\overline{y}_i$ is the mean value of the group, $\overline{y}$ is the mean value of the total measurements, and y_{ij} is the value of

the j^{th} measurement in the i^{th} group. A treatment is defined as a specific combination of factor levels whose effect is to be compared with other treatments.

The *SST* and *SSE* calculated above are used to form two mean squares, *mean square for treatments*, MST, and *mean square for error*, MSE. by dividing the sum of squares by the associated degrees of freedom, the degrees of freedom for treatment, DFT, and the degrees of freedom for error, DFE:

$$MST = SST / DFT \tag{3.6}$$

$$MSE = SSE / DFE \tag{3.7}$$

where $DFT = k - 1$, k is the number of groups of treatments, and $DFE = N - k$, where N is the total number of measurements in all groups.

The test statistic, used in testing the equality of treatment means is:

$$F = MST / MSE \tag{3.8}$$

The critical value is the tabular value of the F distribution, based on the chosen α level and the degrees of freedom, DFT and DFE. The probability of the result assuming the null hypothesis (p) is calculated from the F, DFT, and DFE, which is available at an Internet site (Graphpad.com, 2004).

3.2.6 Data Processing

The effects of pure particles and loaded particles on plant seedling root growth were determined. By comparing the results, conclusions regarding those particle properties that are possibly important for its toxicity were made.

Figure 3.1 presents the comparison strategy design.

The influence of particle chemical composition was evaluated by comparing the effects of particles that are made up of different materials but within same size range. Three metal oxide particle categories are evaluated: Al_2O_3, TiO_2, and SiO_2.

To determine if the particle size is important for particle-mediated injury, the effects of particles made up of the same material were tested in two to three particle size scales: nanometer scale, sub-micrometer scale, and micrometer scale.

The involvement of particle surface area in the particle-induced biological effect was investigated by tests on particles within the same size range but with different specific surface area (m^2/g), and on particles with different size ranges but with the same scale of specific surface area.

3.3 Materials

3.3.1 Particles

Aeroxide® TiO_2 P25 was purchased from Degussa. Aeroxide® Alu C was from Degussa also. Cab-O-Sil® M5 was purchased Cabot. The 161.2 nm hydrophilic spherical silica was locally–made in a conjunction experimental program. The 667.6-nm hydrophilic silica particles were generously given by another research group. The particles were

purchased from a manufacturing company, but the name of the company was not available, according to the research group. The 0.96-μm titania and the 1.00-μm alumina particles were purchased from Atlantic Equipment Engineers (AEE), Inc.

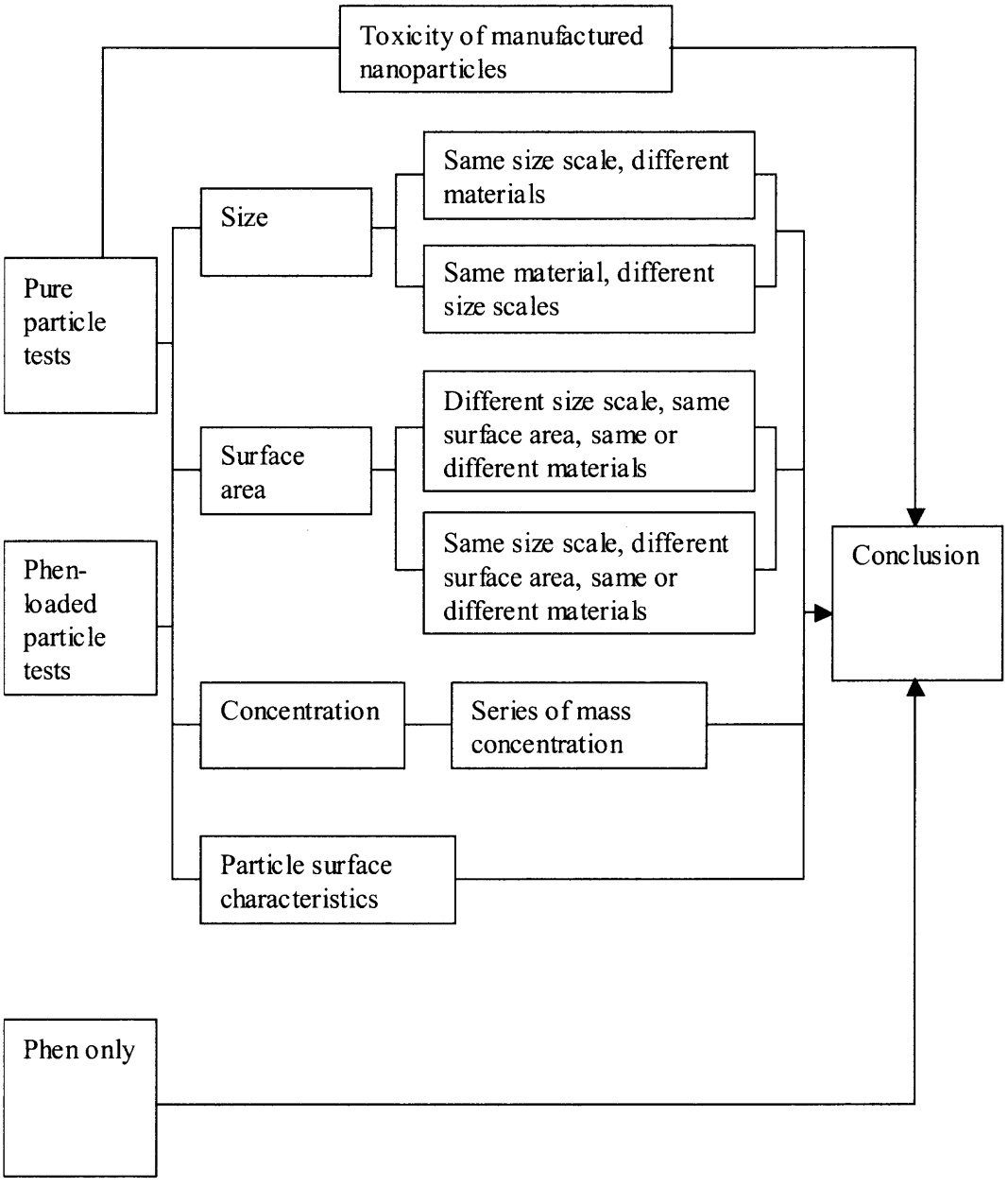


Figure 3.1 Data processing strategy for determination of the most important particle properties that contributes to particle toxicity.

Table 3.2 presents the sizes and specific surface areas of the particles studied in this dissertation.

According to the results from the particle size analysis, the nanoparticles including the 13-nm alumina, 14-nm silica, and 21-nm Titania, appeared in the water mostly as aggregate forms.

Table 3.2 Particle Sizes and Specific Surface Areas

Particle	ID#	Size ± SD	Specific Surface Area m^2/g
Alumina (Al_2O_3)	1	1.00 ± 0.056 μm	1.9449
	2	13 nm[a], size of aggregates 201.0 ± 74.7 nm	103.9681
Silica (SiO_2)	1	Size of aggregates: 667.6 ± 7.89 nm (spherical)	5.7376
	2	161.2 ± 45.6nm[b]	146.9722
	3	14 nm[c], size of aggregates: 215.7 ± 56.3 nm	200
Titania (TiO_2)	1	Size of aggregates0.96 ± 0.009 μm	12.2273
	2	21 nm[d], size of aggregates 119.5 ± 58.2 nm	38.0907

[a]Aeroxide® Alu C from Degussa. The 13 nm is the average diameter of individual particles according to the material datasheet given by the manufacturer.
[b]Locally made in another lab. The particles were made to be mono-dispersed in water. The size of 161.2 nm is thus suggested to be the mean size of the individual particles.
[c]Cab-O-Sil® M5 from Cabot, hydrophilic silica. The 14 nm is the average diameter of individual particles according to the material datasheet given by the manufacturer. The specific surface area data of the 14-nm silica particles was taken from the material datasheet;.
[d]Aeroxide® TiO_2 P25 from Degussa. The 21-nm is the average diameter of individual particles according to the material datasheet given by the manufacturer.

3.3.2 Seeds

Seeds of six plant species: the *Zea mays* (corn), the *Cucumis sativus* (cucumber), the *Glycine max* (soybean), the *Brassica oleracea* (cabbage), the *Avena sativa* (oat), and the *Daucus carota* (carrot) were purchased from Territorial Seed Company (Oregon, USA)

(Figure 3.2). These six species are among the ten plant species recommended by the US EPA (EPA, 1996) to determine ecological effects of pesticides and toxic substances. Seeds were stored in a dry place in the dark at room temperature. Seed germination rates were determined (Table 3.3).

Table 3.3 Germination Rate of the Seeds

Plant species	Germination rate %
Z .mays	92 ± 3
C. sativus	91 ± 2
G. max	87 ± 2
B. cabbage	95 ± 3
A. sativa	83 ± 2
D. carota	88 ± 4

200 seeds/species were cultured with 10 seeds / dish in 5 ml Milli-Q water on filter papers that were placed in 100 × 15 mm Petri dishes, and allowed to germinate in an incubator in the dark at 25 ± 1 °C for 48~72 hrs till the primary roots elongated to about 20~30 mm. Seedlings with primary root less than 6.5 mm were regarded as non-germinated (EPA, 1996). The whole process was repeated three times. The germination rate was calculated and reported as the mean value ± standard deviation.

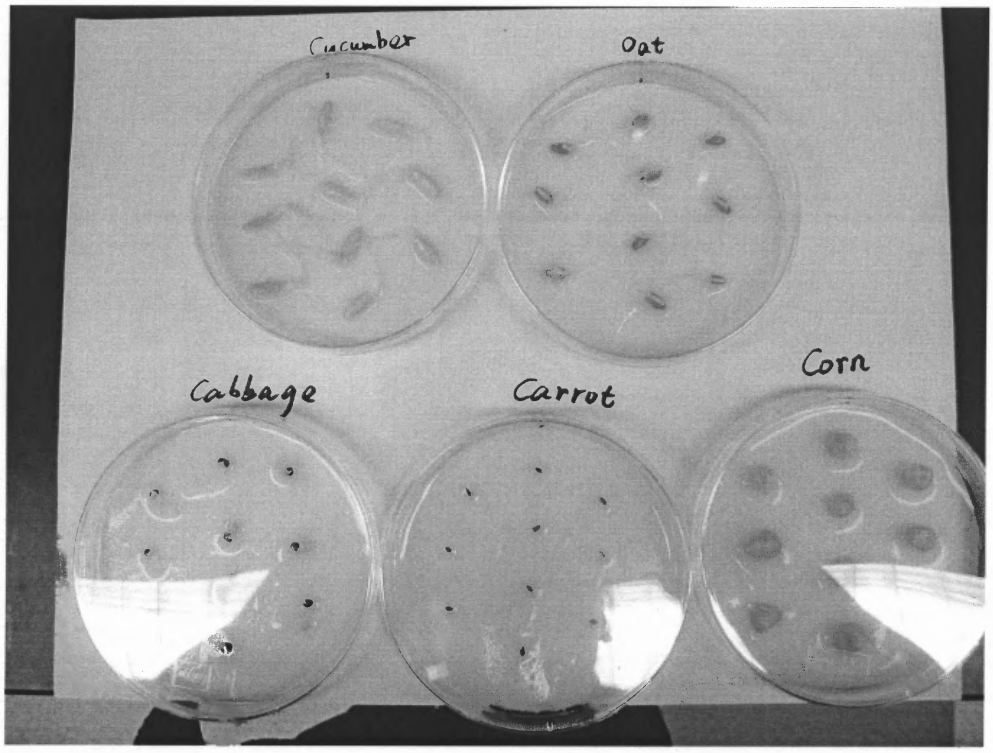


Figure 3.2 Seedlings cultured from the seeds.

CHAPTER 4

EFFECTS OF MANUFACTURED PARTICULATE MATERIALS ON PLANT SEEDLING ROOT GROWTH

The materials and experimental methods used in this study were presented in Chapter 3. The techniques of the root elongation test were applied for the purpose of determination of the effects of the manufactured particles. Six plant species including *Z. mays*, *C. sativus*, *B. oleracea*, *D. carota*, *G. max*, and *A. sativa* were involved in this study. Nanoparticles studied include 13-nm alumina particles, 14-nm hydrophilic silica particles, 21-nm Titania particles, and 161.2-nm hydrophilic silica particles. Submicron and micron particles studied include 667.6-nm hydrophilic silica particles, 0.96-μm titania particles, and 1.00-μm alumina particles.

4.1 13-nm Alumina Particles

4.1.1 24-hr Exposure to the 13-nm Alumina Particles

Root elongation tests were performed on the alumina nanoparticles (Alu-C, Degussa) with individual size of 13 nm and aggregate size of 201.0 ± 74.69 nm. The effect of the alumina particles on plant root growth was investigated at the concentrations of 20 mg/ml, 2 mg/ml, 200 μg/ml, and 20 μg/ml.

The concentrations were determined by a preliminary study, in which, the concentrations of 20 mg/ml, 2 mg/ml, 200 μg/ml, 20 μg/ml, and 2 μg/ml were tested. Neither fatal effect nor 100% inhibitory (RRG = 0.00 in the root elongation test) of the alumina nanoparticles on the seedlings was observed in the concentration range from 20

mg/ml to 2 μg/ml. Typically in toxicology, the highest test concentration is the one that induces 100% effect (for example, 100% of death, or 100% of inhibitory), and the lowest test concentration is the one that does not induce any effect. The highest concentration in this study was then decided as 20 mg/ml, which is the highest concentration tested in the preliminary study. Higher concentration, i.e., 200 mg/ml is unrealistic because this concentration is too high to be a particle suspension. It is a sort of slurry for most of the particles studied in this dissertation. The lowest concentration was decided as the one below a concentration that induced no effect compared to the blank. 200 μg/ml of the 13-nm alumina nanoparticles was not found toxic under the experimental conditions in the preliminary study. The concentration of 20 μg/ml was thus decided as the lowest test concentration for the 13-nm alumina particles.

The plant species used include *Z. mays* (corn), *C. sativus* (cucumber), *B. oleracea* (cabbage), and *D. carota* (carrot). Root elongation (RE) during exposure to the particles was obtained by subtracting the root lengths before the exposure from the root lengths after the exposure (Table 4.1). Relative root growth (RRG) was obtained in order to compare the results among different test batches and different plant species, which was calculated as the ratio of RE of seedlings exposed to particle suspensions to RE of seedlings in the Milli-Q water control (Table 4.1 and Figure 4.1). The relative root growth (RRG) was calculated following the procedure that has been used widely in the literature (Moon et al., 1997; Jorge et al., 2001; Tang et al., 2002; Boscolo et al., 2003; Hoekenga et al., 2003) The results from exposure to the particles and to the controls were statistically analyzed by the *Student's t*-test and *one-way ANOVA* procedure (Table 4.2 and Table 4.3).

Table 4.1 Root Elongation (RE) and Relative Root Growth (RRG) of Plant Seedlings Exposed to Alumina Nanoparticles for 24 hrs in the Dark at 25 ± 1°C

The results are reported as mean ± S.D. and 95% confidence interval.

	Conc.	*Z. mays*	*C. sativus*	*B. oleracea*	*D. carota*
RE (mm)	Blank 1	**13.7 ± 4.6**[a] **12.1 ~ 15.4**	38.4 ± 4.6[c] 37.0 ~ 39.8	17.0 ± 5.5[e] 15.0 ~ 19.0	**8.3 ± 1.6**[g] **7.7 ~ 8.9**
	20 mg/ml	**7.2 ± 1.5** **6.7 ~ 7.8**	26.5 ± 5.2 24.2 ~ 28.8	9.9 ± 3.5 8.6 ~ 11.2	**4.9 ± 1.4** **4.4 ~ 5.4**
	Blank 2	20.0 ± 5.9[b] 17.9 ~ 22.1	**27.0 ± 6.5**[d] **24.8 ~ 29.1**	**18.8 ± 3.8**[f] **17.7 ~ 20.0**	9.8 ± 2.4[h] 9.0 ~ 10.7
	2 mg/ml	15.9 ± 2.0 15.2 ~ 16.6	**22.6 ± 3.8** **21.4 ~ 23.8**	**14.8 ± 2.4** **14.0 ~ 15.5**	8.1 ± 2.2 7.3 ~ 9.0
	200 µg/ml	18.4 ± 3.3 17.2 ~ 19.6	34.9 ± 7.6 32.1 ~ 37.6	14.7 ± 5.0 12.8 ~ 16.5	9.2 ± 3.8 7.7 ~ 10.6
	20 µg/ml	20.7 ± 4.0 19.2 ~ 22.1	37.1 ± 4.9 35.4 ~ 38.9	17.8 ± 7.3 15.2 ~ 20.4	11.1 ± 2.9 10.1 ~ 12.1
RRG	20 mg/ml	**0.53 ± 0.11** **0.44~0.63**	0.64 ± 0.17 0.59~0.69	0.58 ± 0.21 0.49~0.68	**0.59 ± 0.16** **0.52~0.65**
	2 mg/ml	0.79 ± 0.24 0.68~0.90	**0.80 ± 0.14** **0.76~0.85**	**0.78 ± 0.13** **0.73~0.84**	0.83 ± 0.30 0.72~0.93
	200 µg/ml	0.92 ± 0.33 0.78~1.06	0.94 ± 0.16 0.89~1.00	0.87 ± 0.30 0.75~0.98	0.93 ± 0.42 0.77~1.09
	20 µg/ml	1.03 ± 0.32 0.90~1.17	0.96 ± 0.13 0.92~1.01	1.05 ± 0.43 0.91~1.19	1.13 ± 0.43 0.97~1.28

[a] Blank for 20 mg/ml; [b] Blank for 2 mg/ml, 200 µg/ml, and 20 µg/ml; [c] Blank for 20 mg/ml, 200 µg/ml, and 20 µg/ml; [d] Blank for 2 mg/ml; [e] Blank for 20 mg/ml, 200 µg/ml, and 20 µg/ml; [f] Blank for 2 mg/ml; [g] Blank for 20 mg/ml; [h] Blank for 2 mg/ml, 200 µg/ml, and 20 µg/ml.

The growth of seeds and seedlings at an early stage depends on three factors, besides the seeds themselves (e.g. plant species, seed weights, and seed sizes): water, temperature, and air. These three factors were controlled in this study as accurately as possible. It is however impossible to control other factors such as the seed weights and seed sizes. Different plant species in addition have different growth curves. The root elongations of seedlings change dramatically among different plant species and also are different among test batches, as may be seen from Table 4.1. A uniform expression of

Table 4.2 Statistical Analysis Results of the Root Elongation (RE) of Plant Seedlings Exposed to Alumina Nanoparticles for 24 hrs in the Dark at 25 ± 1°C. Part I – Particles vs. Blanks

Results from the *Student's t*-test are reported as the value of t and the value of probability of the result assuming the null hypothesis (p). Results from the *one-way ANOVA* procedure are reported as the value of f, p, and the coefficient of determination (R^2). Statistical difference is reported as p smaller than 0.05.

Concentration	*Z. mays*	*C. sativus*	*B. oleracea*	*D. carota*
20 mg/ml	$t = -7.39, p = 0.000$ $f = 54.5850, p = 0.0001$ $R^2 = 0.5152$	$t = 9.13, p = 0.000$ $f = 83.3367, p = 0.0001$ $R^2 = 0.4104$	$t = 5.88, p = 0.000$ $f = 34.5810, p = 0.0001$ $R^2 = 0.6224$	$t = 8.80, p = 0.000$ $f = 77.486, p = 0.0001$ $R^2 = 0.4281$
2 mg/ml	$t = 3.59, p = 0.001$ $f = 12.9158, p = 0.0007$ $R^2 = 0.8179$	$t = 3.46, p = 0.001$ $f = 11.9859, p = 0.0009$ $R^2 = 0.8538$	$t = 5.92, p = 0.000$ $f = 35.0281, p = 0.0001$ $R^2 = 0.7057$	$t = 2.73, p = 0.009$ $f = 7.447, p = 0.0087$ $R^2 = 0.8747$
200 µg/ml	$t = 1.28, p = 0.206$ $f = 1.6373, p = 0.2058$ $R^2 = 0.9725$	$t = 2.43, p = 0.018$ $f = 5.9096, p = 0.018$ $R^2 = 0.9189$	$t = 1.64, p = 0.106$ $f = 2.7030, p = 0.1058$ $R^2 = 0.9540$	$t = 0.802, p = 0.426$ $f = 0.6438, p = 0.4258$ $R^2 = 0.9884$
20 µg/ml	$t = -0.501, p = 0.618$ $f = 0.2514, p = 0.618$ $R^2 = 0.9957$	$t = 1.15, p = 0.255$ $f = 1.3155, p = 0.2554$ $R^2 = 0.9816$	$t = -0.492, p = 0.625$ $f = 0.2418, p = 0.6248$ $R^2 = 0.9960$	$t = -1.83, p = 0.072$ $f = 3.3552, p = 0.0721$ $R^2 = 0.9453$

data must be used in order to make the data comparable among different test batches and plant species. The relative root growths (RRG) were obtained for this purpose as the ratios between the mean values of the RE results for the seedlings exposed to particle suspensions to those for the seedlings grown in the Milli-Q water. RRG was used for comparison between two plant species or two test batches instead of RE, whereas RE was used for comparison between two concentrations of particle suspensions tested within the same test batch. That is the fact that for *Z.mays*, and *D.carota*, the RE values were used for comparing the results between 2 mg/ml, 200 μg/ml, and 20 μg/ml, and the RRG values were used for comparing 20 mg/ml with the other three concentrations. The statistical analysis performed for the *C. sativus* and the *B. oleracea* used the RE values the comparison between 20 mg/ml, 200 μg/ml, and 20 μg/ml, and the RRG results for evaluation of the differences between the effects on root growth of 2 mg/ml and the other three concentrations. Table 4.3 gives the results.

The relative root growth remained constant, although root elongations during the exposure period changed with the plant species and the test batches. The examples are, when exposed to the 2-mg/ml 13-nm alumina particle suspensions, the primary root of seedlings elongated from 8.1 mm for *D. carota* to 22.6 mm for *C. sativus*, whereas at the same concentration, RRG is from 0.78 for *B.oleracea* to 0.83 for *D. carota*. RE of *C. sativus* seedlings, when tested at a different time, was 26.5 mm exposed to 20 mg/ml of particle suspensions, which was even larger than the 22.6 mm of RE of seedlings exposed to 2 mg/ml of particle suspensions at an earlier time, even though It is frequently observed that the RE values decrease with an increase of particle suspension

Table 4.3 Statistical Analysis Results of the Root Elongation (RE) and the Relative Root Growth (RRG) of Plant Seedlings Exposed to Alumina Nanoparticles for 24 hrs in the Dark at 25 ± 1°C. Part II – Comparison between Different Concentrations of Particle Suspensions, and between Different Plant Species

Results from the *Student's t*-test are reported as the value of t and p. Results from the *one-way ANOVA* procedure are reported as the value of f, p, and R^2. Statistical difference is reported as p smaller than 0.05.

Comparison	*Z. mays*	*C. sativus*	*B. oleracea*	*D. carota*
20 mg/ml vs. 2 mg/ml	p_1[a] = 0.000 p_2[b] = 0.0001 R^2 = 0.3658	p_1 = 0.000 p_2 = 0.0003 R^2 = 0.7834	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7376	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7102
20 mg/ml vs. 200 µg/ml	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.3723	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7203	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7632	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7319
20 mg/ml vs. 20 µg/ml	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.3802	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.4730	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.6730	p_1 = 0.000 p_2 = 0.0001 R^2=0.4246
2 mg/ml vs. 200 µg/ml	p_1 = 0.001 p_2 = 0.0006 R^2 = 0.8164	p_1 = 0.110 p_2 = 0.1104 R^2 = 0.9600	p_1 = 0.123 p_2 = 0.1226 R^2 = 0.9658	p_1 = 0.243 p_2 = 0.2433 R^2 = 0.9723
2 mg/ml vs. 20 µg/ml	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.6240	p_1 = 0.000 p_2 = 0.0003 R^2 = 0.8161	p_1 = 0.000 p_2 = 0.0004 R^2 = 0.8328	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.7490
200 µg/ml vs. 20 µg/ml	p_1 = 0.020 p_2 = 0.0203 R^2 = 0.9106	p_1 = 0.179 p_2 = 0.2554 R^2 = 0.9816	p_1 = 0.064 p_2 = 0.0636 R^2 = 0.9409	p_1 = 0.036 p_2 = 0.0355 R^2 = 0.9221
Four plant species	At 2 mg/ml, 200 µg/ml, and 20 µg/ml, no significant difference of seedling RRG was found among the four plant species. At 20 mg/ml, significant difference exists between the RRG results of *C. sativus* seedling and seedlings of other two species: *Z.mays* and *D. carota*.			

[a] Calculated from the *Student's t-test*; [b] Calculated from the *one-way ANOVA* procedure

Results are expressed as mean value ± S.D., not error bars.

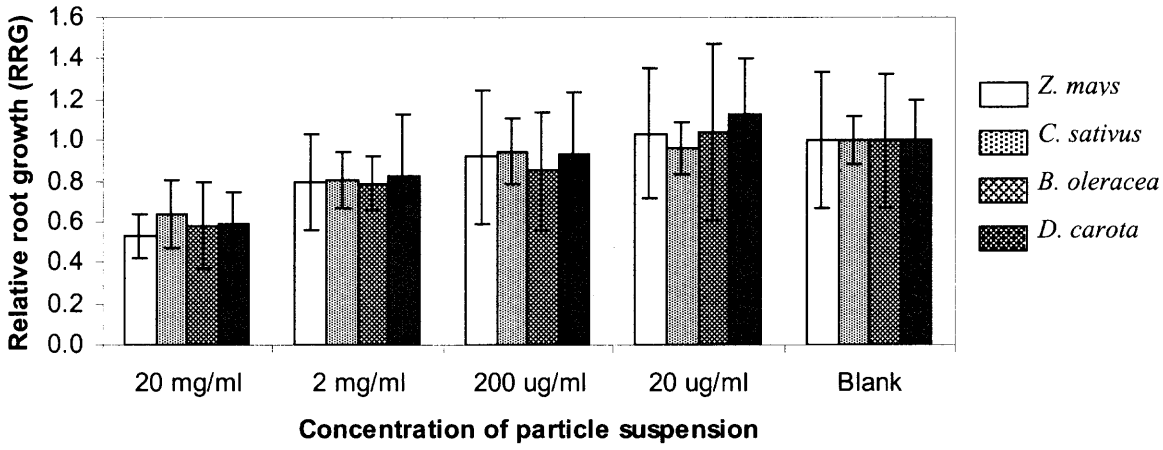


Figure 4.1 Relative root growth of seedlings exposed to 13-nm alumina particles for 24 hrs in the dark at 25 ± 1°C –evaluated by four plant species.

concentrations within the range of concentrations studied. The data always decrease with the increase of the concentrations in the case of RRG.

It is obvious from the coefficient of determination (R^2) presented in Table 4.2 that the root growth of the seedlings exposed to the particle suspensions approaches that of seedlings cultured in the control as the concentration decreases: in the case of *Z. mays*, the R^2 was 0.5152 at 20 mg/ml, 0.8179 at 2 mg/ml, 0.9725 at 200 μg/ml, and 0.9957 at 20 μg/ml; in the case of *C. sativus*, the R^2 was 0.4104 at 20 mg/ml, 0.8538 at 2 mg/ml, 0.9189 at 200 μg/ml, and 0.9816 at 20 μg/ml; in the case of *B. oleracea*, the R^2 was 0.6224 at 20 mg/ml, 0.7057 at 2 mg/ml, 0.9540 at 200 μg/ml, and 0.9960 at 20 μg/ml; and for *D. carota*, the R^2 was 0.4281 at 20 mg/ml, 0.8747 at 2 mg/ml, 0.9884 at 200 μg/ml, and 0.9453 at 20 μg/ml. There is a statistical difference between the effects of blank and concentrations of 20 mg/ml and 2 mg/ml. A statistical difference existed between the effects of 20 mg/ml and either 2 mg/ml, 200 μg/ml or 20 μg/ml, with

$p<<0.05$ in all circumstances. 2 mg/ml and 200 μg/ml at the same time have no statistically different effects on the root growth, although 200 μg/ml was not found toxic when compared to the blank. Significant difference however was found between the effects of 2 mg/ml and 20 μg/ml. The results suggest that 20 mg/ml and 2 mg/ml of the 13-nm alumina is toxic to the root growth of the seedlings, and the observed phytoxicity of the alumina nanoparticles decreases with a decrease of the concentration of the particle suspension.

Mean values of the RRGs were calculated from the RRG results for the four plant species to evaluate the toxicity of 13 nm alumina particles. The RRG results obtained from the tests using the four plant species were first statistically analyzed using the *one-way ANOVA*, which determines whether there is a difference among multiple mean values. There is no statistical difference among the results for the four plant species at 2 mg/ml, 200 μg/ml, or 20 μg/ml of the particle suspensions, with p = 0.7189, 0.6911, and 0.1931 for 2 mg/ml, 200 μg/ml, and 20 μg/ml, respectively. At 20 mg/ml however, the RRG result obtained from the test using the *C. sativus* showed a difference from what obtained through the tests using two other plant species: the *Z. mays*, and the *D. carota*. The mean value of RRG for 20 mg/ml of particle suspension thus was calculated from the other three plant species: the *Z. mays*, the *D. carota*, and the *B. oleracea*. The dose-effect relationship of 13 nm alumina particles was evaluated by a linear regression, which was performed based on the logarithm of concentration of particle suspensions and the mean value of the RRGs (Figure 4.2).

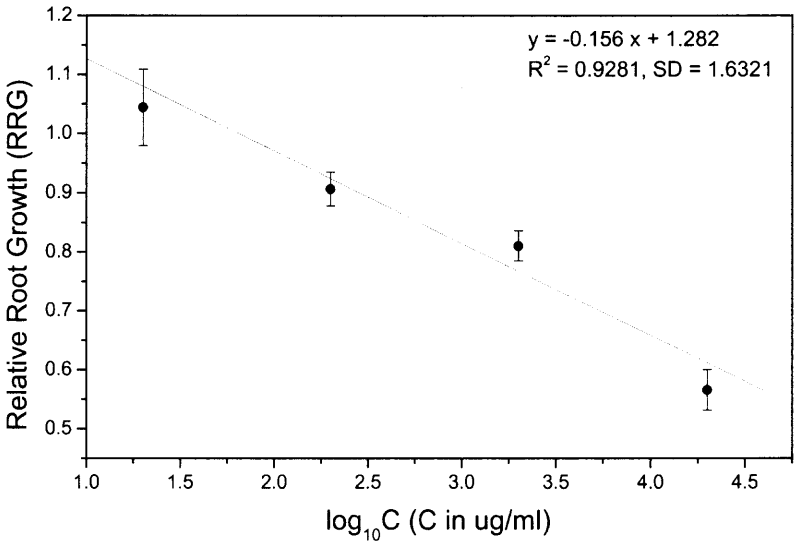


Figure 4.2 Relative root growth of seedlings exposed to 13-nm alumina particles in the dark at 25 ± 1°C for 24 hrs – mean value of the RRG of seedlings of four plant species. Linear regression evaluated by logarithm of particle suspension concentrations.

The linear regression was performed according to the standard data analysis procedure in toxicology, and can be referenced in published studies (Ryan et al., 2004; Xu et al., 2004; Kmetic et al., 2003; McDonald et al., 1996; and Lee et al., 1989). The toxicity threshold value, for example, LC_{50} (the median lethal concentration), IC_{10} (the 10% inhibitory concentration), and LD_{90} (the 90% lethal dose), were often calculated from the linear regression between the different levels of acute toxic effect that were determined in a toxicity test (e.g. 0% lethal, 9% lethal, 21% lethal, 73% lethal, and 92% lethal) and concentrations/doses or a function (typically is a logarithm) of concentrations or doses of the toxic substances.

The inhibition effect of 13-nm alumina particles at 200 μg/ml compared to the blank is around 1.09 (1.06 to 1.15, the number was calculated as the ratio of RRG from

blank to RRG from particle suspension), 1.25 (1.20 to 1.28) fold at 2 mg/ml, and 1.71 (1.56 to 1.89) fold at 20 mg/ml. The linear regression evaluated by the logarithm of particle suspension concentrations ($\log_{10}$C, C in μg/ml) to RRG [Fig 4.2.1(b)] suggests that the root growth inhibition effect of 13-nm alumina particles is dose dependent, with the R^2 = 0.9281. The high coefficient of determination means that the linear fit for the data analysis is feasible, and the RRG values are highly related to the logarithm of the particle suspension concentrations. The negative slope (-0.156) indicates the RRG of seedlings exposed to the 13-nm alumina particles decreases with the increase of the logarithm of the particle concentration, which means that the phytotoxic effect of 13-nm alumina particles on the seedling root growth increases with the increase of the particle concentration. The linear regression analysis suggests that the inhibitory effect of the 13-nm alumina particles is dose-dependent. The fact that at 200 μg/ml, the alumina particles have no effect on seedling root growth with p larger than 0.05, indicates the existence of a phytotoxicity threshold value for 13-nm alumina particles.

4.1.2 72-hr Exposure to the 13-nm Alumina Particles

C. sativus seedlings were exposed to 2 mg/ml of alumina nanoparticle suspensions for 24 hrs, 48 hrs and 72 hrs. *B. oleracea* seedlings were exposed to 20 mg/ml of alumina nanoparticle suspensions for 24 hrs, and 48 hrs. The pH values of particle suspensions in the Petri dishes were determined by pH test strips at the end of each test duration. All of the pH values were found to be around 7 (6.5 to 7.0). RE values of seedlings were obtained (Table 4.4).

The RRG of *C. sativus* seedlings exposed to 2 mg/ml of particle suspensions is 0.90 at the end of the first 24 hrs of exposure, compared to 0.93 and 0.98 at the end of the

second 24 hrs and the third 24 hrs of exposure. *B. oleracea* seedlings were exposed to 20 mg/ml of particle suspensions. The RRG was observed as 0.57 at the end of the first 24 hrs of exposure, and 0.61 at the end of the second 24 hrs of exposure (Figure 4.3).

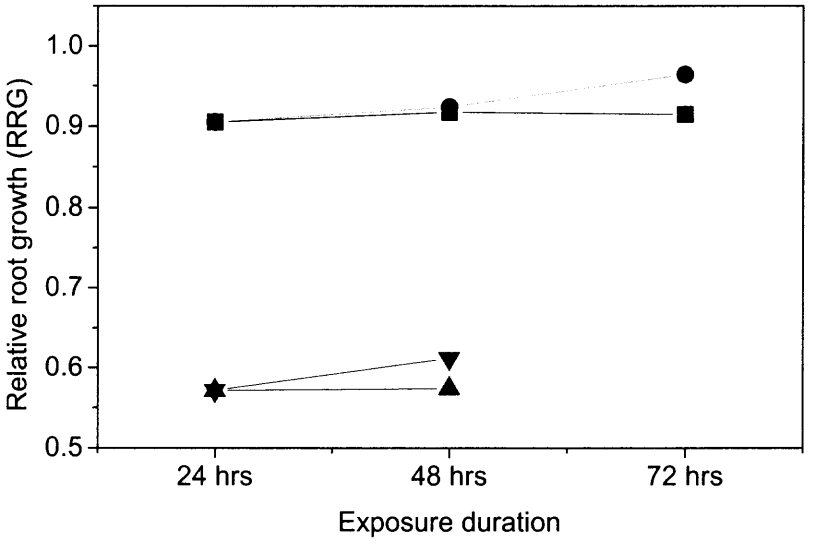
Table 4.4 Root Elongation (RE) of *C. sativus* Seedlings Exposed to 2 mg/ml of Alumina Nanoparticle Suspensions for 24 hrs, 48 hrs, and 72hrs, and of *B. oleracea* Seedlings Exposed to 20 mg/ml of Alumina Nanoparticle Suspensions for 24 hrs and 48 hrs

The result reported is the mean value ± S.D. Statistical analysis was performed by the *Student's t*-test and *one-way ANOVA* by comparing RE from blank and particle suspensions, as well as RE among particle suspensions. Statistical difference is reported as the p smaller than 0.05.

	24 hours	48 hours		72 hours	
		Total	24 hours	Total	24 hours
Blank	39.1 ± 5.2	70.9 ± 12.4	31.8 ± 11.7	94.2 ± 12.9	23.3 ± 7.2
	37.2 ~ 41.0	66.4 ~ 75.3	27.6 ~ 36.0	89.6 ~ 98.8	20.7 ~ 25.9
C. sativus	35.4 ± 5.2	65.0 ± 10.7	29.5 ± 8.2	87.6 ± 12.8	22.6 ± 9.2
(2 mg/ml)	33.5 ~ 37.2	61.1 ~ 68.7	26.6 ~ 32.4	82.9 ~ 92.1	19.3 ~ 25.9
Compared	p_1 =0.008	p_1 =0.050	p_1 =0.386	p_1 =0.051	p_1 =0.760
to the blank	p_2 = 0.0078	p_2 = 0.0500	p_2 = 0.3860	p_2 = 0.0506	p_2 = 0.7598
	R^2 = 0.8841	R^2 = 0.9354	R^2 = 0.9870	R^2 = 0.9357	R^2 = 0.9983
Blank	17.0 ± 5.4	31.3 ± 8.7	13.1 ± 6.9		
	15.1 ~ 18.9	27.8 ~ 34.8	10.4 ~ 15.9		
B. oleracea	9.9 ± 3.5	18.0 ± 5.7	8.0 ± 3.8		
(20 mg/ml)	8.6 ~ 11.2	15.9 ~ 20.0	6.6 ~ 9.4		
Compared	p_1 =0.000	p_1 =0.000	p_1 =0.001		
to the blank	p_2 = 0.0001	p_2 = 0.0001	p_2 = 0.0012		
	R^2 = 0.6224	R^2 = 0.5298	R^2 = 0.8153		

It is observed and considered reasonable that the root elongation of seedlings decreases as the exposure or culture duration increases. The statistical analysis that was based on the RRG results shows no statistically significant difference among the RRG values of seedlings exposed to 2 mg/ml or 20 mg/ml alumina nanoparticles suspensions in the first 24 hrs of exposure and after the first 24 hrs of exposure. The statistical analysis performed for the exposure of the *C. sativus* seedlings to 2 mg/ml of alumina

nanoparticle suspensions shows that the value of p is 0.5860 for 24hr-48hr-72hr, and 0.4255 for 1st 24hr-2nd 24hr-3rd 24hr. The statistical analysis performed for the exposure of the *B. sativus* seedlings to 20 mg/ml of particle suspensions shows that the p is 0.9992 for 24hr-48hr, and 0.9970 for 1st 24hr-2nd 24hr. All of the p values are much larger than 0.05, indicating there is no statistically significant difference between the RRG results that were being compared. Compared to the blank control, for *C. sativus* seedlings that were exposed to 2 mg/ml of the particle suspensions, the p value is 0.0078 in the first 24



- ■ RRG of seedlings exposed to 2 mg/ml of particle suspensions for 24 hrs, 48 hrs, and 72 hrs
- ● RRG of seedlings exposed to 2 mg/ml of particle suspensions for the first 24 hrs, the second 24 hrs and the third 24 hrs
- ▲ RRG of seedlings exposed to 20 mg/ml of particle suspensions for 24 hrs and 48 hrs
- ▼ RRG of seedlings exposed to 20 mg/ml of particle suspensions for the first 24 hrs and the second 24 hrs

Figure 4.3 RRG of *C. sativus* and *B. oleracea* seedlings exposed to 2 mg/ml or 20 mg/ml of alumina nanoparticle suspensions of 24 hrs, 48 hrs, and 72 hrs.

hrs of exposure, 0.3860 in the second 24 hrs of exposure, and 0.7598 in the third hrs of exposure. The R^2, which evaluates the correlation between two samples, keeps increasing from 0.8841 in the first 24 hrs, 0.9870 in the second 24 hrs, to 0.9983 in the third 24 hrs, suggesting that in the 1st 24 hrs, the particles inhibited the root growth, whereas after the 1st 24 hrs, the root growth of the seedlings exposed to the particle suspensions was not statistically different than that of the seedlings cultured in the blank. The situation was different for *B. oleracea* seedlings that were exposed to 20 mg/ml of the particle suspensions, the p value is 0.000 and 0.001 for the first 24 hrs and the second 24 hrs respectively, the R^2 increased from 0.6224 in the first 24 hrs to 0.8153 in the second 24 hrs. The increasing R^2 suggests the root growth of the seedlings exposed to the particles approaches that of the seedlings cultured in the blank. If the toxic effect of the particles is time-dependent, which means that the toxic effect increases with the increase of exposure duration, the RRG values should significantly decrease with the exposure duration. The value of R^2 that evaluates the correlation between the RE result from the particle suspensions and the RE results from the blank control should decrease with the exposure duration. The RRG results observed in this study suggest that the most toxic effect of alumina nanoparticles is present at the first 24 hrs, and that the effect of 13-nm alumina particles on plant seedling root growth is not time dependent, relative to the 72-hr time period of these studies.

4.1.3 Water Treatment Test on the 13-nm Alumina Particles

This test was designed to investigate whether the inhibition effect of the 13-nm alumina particles is reversible. The effect is not adverse if it is reversible, and the particles are not toxic, by definition.

The *C. sativus* seedlings were used in this test. The seedlings were exposed to 2 mg/ml of the particle suspensions for 24 hrs. The seedlings were taken out at the end of the exposure and rinsed with Milli-Q water from a plastic wash bottle to remove the particles. The rinse was repeated till the effluent was visibly clear to the eye instead of cloudy as it originally appeared (this rinse required approximate 500 ml of Milli-Q water). They were then cultured for 24 hrs on a filter paper that was soaked in 5 ml Milli-Q water in Petri dishes. The RE and RRG of seedlings were obtained, and compared to the blank controls and the results from the first 24 hrs of exposure (Table 4.5).

The RE of the seedlings changed during the first 24 hrs and the second 24 hrs (for example, $p = 0.0011$ for the blank). The RRG ratios of the seedlings however were similar, either during the first 24 hrs and the second 24 hrs ($p = 0.9992$ for the particles), or during the first 24 hrs and the whole 48 hrs ($p = 0.9998$ for the particles). The mean value of RE of the seedlings exposed to the 2 mg/ml of the particle suspensions was significantly smaller than that of the seedlings in the blank control in the first 24 ($p = 0.0016$). It was also significantly smaller than that of the seedlings in the blank control in the second 24 hrs and the whole 48 hrs ($p = 0.0044$ and 0.0002 for the second 24 hrs and the whole 48 hrs, respectively). The data signifies that first, the root growth of the seedlings was still inhibited after the particles have been removed for 24 hrs; second, the inhibition effect of the 13-nm alumina particles is an adverse effect on plant seedling root growth; and third, the 2 mg/ml of the 13-nm alumina particle suspensions are toxic to the root growth of the seedlings. It has been determined at the same time, when compared to the results from the test for 48-hr exposure, that after the same duration of incubation, the RRG of the seedlings in the water treatment test was not significantly smaller than that of

the seedlings in the particle suspensions (p = 0.086 for the second 24 hrs, and 0.072 for the whole 48 hrs), indicating that the water treatment had a similar effect to the long time exposure.

Table 4.5 The RE and RRG Results of the *C. sativus* Seedlings that were Exposed to 2 mg/ml of 13-nm Alumina Particle Suspensions (Particles) in the Dark at 25 ± 1 °C for 24 hrs, and then Cultured in Milli-Q Water for Another 24 hrs in the Same Incubator

The results are reported as the mean value ± S.D. Significant difference is reported as the $p < 0.05$.

		RE mm	RRG
First 24 hrs (Exposure)	Blank	46.3 ± 12.6, 41.8 ~ 50.8	1.00 ± 0.27, 0.90 ~ 1.10
	Particle	37.3 ± 7.7, 34.4 ~ 40.1	0.80 ± 0.17, 0.74 ~ 0.86
		p_1[a] = 0.0016, p_2[b] = 0.0016 R^2 = 0.8389	
Second 24 hrs (Water treatment)	Blank	36.7 ± 8.9, 33.5 ~ 39.8	1.00 ± 0.24, 0.91 ~ 1.09
	Particle	30.0 ± 8.6, 26.8 ~ 33.1	0.82 ± 0.23, 0.73 ~ 0.90
		p_1 = 0.0044, p_2 = 0.00443 R^2 = 0.8320	
First 24 hrs vs. Second 24 hrs	Blank	p_1 = 0.0011, p_2 = 0.00114 R^2 = 0.8320	-
	Particle	-	p_1 = 0.830, p_2 = 0.8304 R^2 = 0.9992
48 hrs	Blank	83.0 ± 14.8, 77.6 ~ 88.3	1.00 ± 0.24, 0.91 ~ 1.09
	Particle	67.2 ± 15.4, 61.6 ~ 72.8	0.81 ± 0.19, 0.74 ~ 0.88
		p_1 = 0.0002, p_2 = 0.00019 R^2 = 0.7815	
First 24 hrs vs. 48 hrs	Blank	p_1 = 0.000, p_2 = 0.0001 R^2 = 0.3523	-
	Particle	-	p_1 = 0.914, p_2 = 0.9135 R^2 = 0.9998

[a] Calculated from the *Student's t-test*;
[b] Calculated from the *one-way ANOVA* procedure.

4.1.4 Conclusions – the Effect of 13-nm Alumina Particles on Root Growth

The results from the root elongation test using four plant species and 13 nm alumina particle suspensions with concentrations of 20 mg/ml, 2 mg/ml, 200 μg/ml, and 20 μg/ml suggest that:

1) The inhibition effect of the alumina nanoparticles presents itself at concentrations equal to or larger than 2 mg/ml.

2) The inhibitory effect of the alumina nanoparticles is dose-dependent, with the IC_{10} (concentration that produces 10% of the inhibitory effect) is determined as 281 μg/ml by the linear regression equation.

3) The inhibitory effect of the alumina nanoparticles is not time-dependent, and occurs during the first 24 hrs of exposure, and

4) The inhibitory effect of the alumina nanoparticles is irreversible, indicating that the effect is toxic effect, and the alumina nanoparticles are phytotoxic.

4.2 14-nm Hydrophilic Silica Particles

4.2.1 24-hr Exposure to the 14-nm Hydrophilic Silica Particles

The 14-nm hydrophilic silica particles were obtained from Cabot. The aggregate size of the particles is 215.7 ± 56.3 nm (analyzed by Coulter N4+, Table 3.1). Phytotoxicity of the particles was investigated by a root elongation test using three plant species: *B. oleracea* (cabbage), *D. carota* (carrot), and *C. sativus* (cucumber). A series of concentrations was tested including 20 mg/ml, 2 mg/ml, 200 μg/ml, and 20 μg/ml. The determination procedure is similar to that had been done for the 13-nm alumina particles (see Section 4.1.1). The RE values were obtained by comparing the root lengths before and after exposure. Relative root growth (RRG) was calculated (Table 4.6 and Figure 4.4). The statistical analysis was performed by the *Student's t*-test and *one-way ANOVA* (Table 4.7 and Table 4.8).

The mean value of RE of plant seedlings that were exposed to 20 mg/ml of the particles is 1.15 (*D. carota*) to 1.22 (*C. sativus*) fold greater, compared to the RE from the blanks. The root elongation of seedlings of every plant species decreases with the decrease of the concentration, and approaches the RE results from the blanks at the

particle concentration of 20 μg/ml. Statistical analysis that compared the RE results from particle suspensions to the results from the blanks (Table 4.7) shows that the difference is significant between the RE values of the seedlings exposed to 20 mg/ml of the 14 nm hydrophilic silica particles and the REs of the seedlings cultured in the blank control, whereas there is no statistical difference between the root growth of seedlings exposed to 2 mg/ml, 200 μg/ml, or 20 μg/ml and the blank controls exists.

Table 4.6 Root Elongation (RE) and Relative Root Growth (RRG) of Plant Seedlings Exposed to 14-nm Hydrophilic Silica Nanoparticles for 24 hrs in the Dark at 25 ± 1°C

The results are reported as mean ± S.D. and 95% confidence interval.

	Concentration	*B. oleracea*	*D. carota*	*C. sativus*
RE (mm)	Blank	17.0 ± 5.5	12.8 ± 3.7	17.4 ± 6.0
		15.0 ~ 19.0	11.6 ~ 14.1	15.0 ~ 19.8
	20 mg/ml	19.9 ± 5.0	14.8 ± 3.3	21.2 ± 6.6
		18.0 ~ 21.8	13.5 ~ 16.0	18.3 ~ 24.1
	2 mg/ml	19.0 ± 6.3	12.8 ± 3.0	18.7± 7.8
		16.8 ~ 21.2	11.6 ~ 14.1	15.4 ~ 22.0
	200 μg/ml	16.8 ± 6.0	12.7 ± 2.7	18.4 ± 5.3
		14.7 ~ 18.9	11.2 ~ 14.2	16.0 ~ 20.9
	20 μg/ml	16.3 ± 4.2	12.3 ± 3.9	18.2 ± 6.1
		14.5 ~ 18.2	10.9 ~ 13.7	15.7 ~ 20.8
RRG	20 mg/ml	1.17 ± 0.29	1.15 ± 0.26	1.22 ± 0.38
		1.06~1.28	1.05 ~ 1.25	1.06 ~ 1.38
	2 mg/ml	1.12 ± 0.37	1.00 ± 0.23	1.07 ± 0.45
		0.99~1.25	0.90 ~ 1.10	0.90 ~ 1.25
	200 μg/ml	0.99 ± 0.35	0.99 ± 0.21	1.06 ± 0.30
		0.86~1.11	0.87 ~ 1.11	0.90 ~ 1.22
	20 μg/ml	0.97 ± 0.25	0.96 ± 0.30	1.05 ± 0.35
		0.86~1.07	0.85 ~ 1.07	0.90 ~ 1.20

Table 4.7 Statistical Analysis Results of the Root Elongation (RE) of Plant Seedlings Exposed to Silica Hydrophilic Nanoparticles for 24 hrs in the Dark at 25 ± 1°C. Part I – Particles vs. Blanks

Results from the *Student's t*-test are reported as the value of *t* and the value of probability of the result assuming the null hypothesis (*p*). Results from the *one-way ANOVA* procedure are reported as the value of *f*, *p*, and the coefficient of determination (R^2). Statistical difference is reported as *p* smaller than 0.05.

Concentration	*B. oleracea*	*D. carota*	*C. sativus*
20 mg/ml	p_1[a] = 0.036	p_1 = 0.040	p_1 = 0.054
	p_2[b] = 0.0356	p_2 = 0.0397	p_2 = 0.0536
	R^2 = 0.9248	R^2 = 0.8622	R^2 = 0.9121
2 mg/ml	p_1 = 0.193	p_1 = 0.985	p_1 = 0.538
	p_2 = 0.1929	p_2 = 0.9849	p_2 = 0.5376
	R^2 = 0.9704	R^2 = 1.0000	R^2 = 0.9909
200 µg/ml	p_1 = 0.897	p_1 = 0.887	p_1 = 0.560
	p_2 = 0.8966	p_2 = 0.8869	p_2 = 0.5599
	R^2 = 0.9997	R^2 = 0.9996	R^2=0.9912
20 µg/ml	p_1 = 0.627	p_1 = 0.606	p_1 = 0.636
	p_2 = 0.6266	p_2 = 0.6059	p_2 = 0.6357
	R^2 = 0.9958	R^2 = 0.9951	R^2 = 0.9947

[a] Calculated from the *Student's t-test*

[b] Calculated from the *one-way ANOVA* procedure

Table 4.8 Statistical Analysis Results of the RE or RRG Values of Plant Seedlings Exposed to Silica Hydrophilic Nanoparticles in the Dark at 25 ± 1°C for 24 hrs. Part II – Comparison between Different Concentrations and Plant Species

	B. oleracea	*D. carota*	*C. sativus*
20 mg/ml vs. 2 mg/ml	p_1[a] = 0.547 p_2[b] = 0.5472 R^2 = 0.9938	p_1 = 0.022 p_2 = 0.0224 R^2 = 0.9133	p_1 = 0.272 p_2 = 0.2723 R^2 = 0.9692
20 mg/ml vs. 200 µg/ml	p_1 = 0.032 p_2 = 0.0323 R^2 = 0.9234	p_1 = 0.026 p_2 = 0.0258 R^2 = 0.9007	p_1 = 0.246 p_2 = 0.2461 R^2 = 0.9479
20 mg/ml vs. 20 µg/ml	p_1 = 0.004 p_2 = 0.0044 R^2 = 0.8662	p_1 = 0.012 p_2 = 0.0122 R^2 = 0.8947	p_1 = 0.139 p_2 = 0.1390 R^2 = 0.9461
2 mg/ml vs. 200 µg/ml	p_1 = 0.167 p_2 = 0.1673 R^2 = 0.9674	p_1 = 0.886 p_2 = 0.8857 R^2 = 0.9996	p_1 = 0.923 p_2 = 0.9231 R^2 = 0.9997
2 mg/ml vs. 20 µg/ml	p_1 = 0.063 p_2 = 0.0632 R^2 = 0.9408	p_1 = 0.586 p_2 = 0.5858 R^2 = 0.9948	p_1 = 0.838 p_2 = 0.8381 R^2 = 0.9990
200 µg/ml vs. 20 µg/ml	p_1 = 0.752 p_2 = 0.7522 R^2 = 0.9982	p_1 = 0.710 p_2 = 0.7102 R^2 = 0.9970	p_1 = 0.725 p_2 = 0.7251 R^2 = 0.9997
20 mg/ml		p_2 = 0.7370	
2 mg/ml		p_2 = 0.4098	
200 µg/ml		p_2 = 0.6960	
20 µg/ml		p_2 = 0.5030	
All together		p_2 = 0.0312	

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

To examine whether the different particle concentrations have different effects on root growth, the RE results from the four concentrations were compared (Table 4.8). The RRG values from the three plant species were statistically evaluated to determine if there is difference between the RRG values of seedlings from different plant species. Statistically, it was found that the effect of 20 mg/ml of the particles is significantly

different than that of other three concentrations. No difference was found among the three species within any of the four concentrations, with p from 0.4098 to 0.7370.

The results demonstrate that at 20 mg/ml, the 14-nm hydrophilic silica particles can enhance the root growth of seedlings significantly. This phenomenon was repeatable in tests using different plant species.

The effect of 14-nm hydrophilic silica particles on seedling root growth was reported as the mean value of the RRG ratios from the tests using the three plant species: the *B. oleracea*, the *D. carota*, and the *C. sativus*. The linear regression performed was based on the logarithm of the concentrations of the particle suspensions and the respective resulting 1/RRG values (Figure 4.4). The 1/RRG was used in this linear regression because the effect of the particles is root growth enhancement, not inhibition. Accordingly, the 10% of the effect (i.e., 1/RRG = 0.90) is actually 1.11 fold larger than the root growth in the blank.

A high coefficient of determination, 0.9497, exists between the two parameters, which suggests that the linear fit is feasible for the data analysis, and the enhancement effect of the silica nanoparticles on seedling root growth is closely related to the logarithm of the particle concentrations. The negative slop (-0.0549) suggests the decrease of 1/RRG with the increase of the logarithm of the particle concentrations. The smaller 1/RRG indicates larger enhancement effect in this case of the 14-nm hydrophilic silica particles. The negative slop thus suggests the increase in the enhancement effect of the particles with larger concentrations of the particle suspensions. The enhancement effect of the 14-nm hydrophilic silica particles is dose-dependent under these conditions.

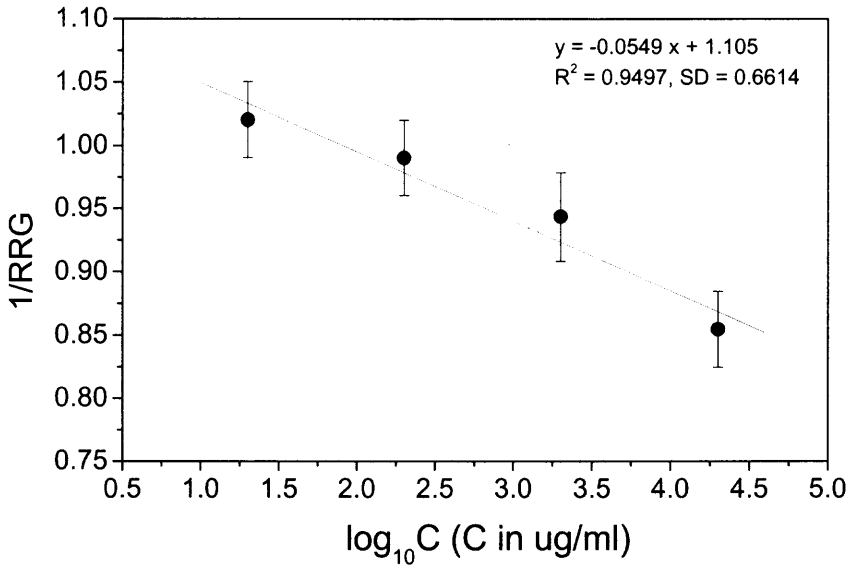


Figure 4.4 Relative root growth of seedlings exposed to 14 nm hydrophilic silica particles in the dark at 25 ± 1°C for 24 hrs – mean value of the RRG of seedlings of three plant species. Linear regression evaluated by logarithm of particle suspension concentrations.

The toxic effect of a chemical is defined as "any adverse effect of the chemical on a living organism". Further, the definition of adverse effect is "an abnormal or harmful effect to an organism caused by exposure to a chemical", and it is indicated by "...altered living activities such as body or organ weight, and enzyme levels etc" (hyperdictionary.com, 2004). The enhancement of root growth brought on by the hydrophilic silica particles might be regarded as an adverse effect of the particles from this definition. The difference between an adverse effect and a non-adverse effect is whether the change resulting from the effect is reversible or not. A non-adverse effect will usually be reversed when the organism is no longer being exposed to the chemical.

The seedlings were treated by Milli-Q water after 24 hrs of exposure to the particles to determine whether the enhancement effect of the silica nanoparticles is reversible, which is discussed in Section 4.2.3.

4.2.2 48-hr Exposure to the 14-nm Hydrophilic Silica Particles

Seedlings of the *B. oleracea* were exposed to 20 mg/ml of the silica nanoparticles for 24 hrs and 48 hrs. Figure 4.5 shows the results.

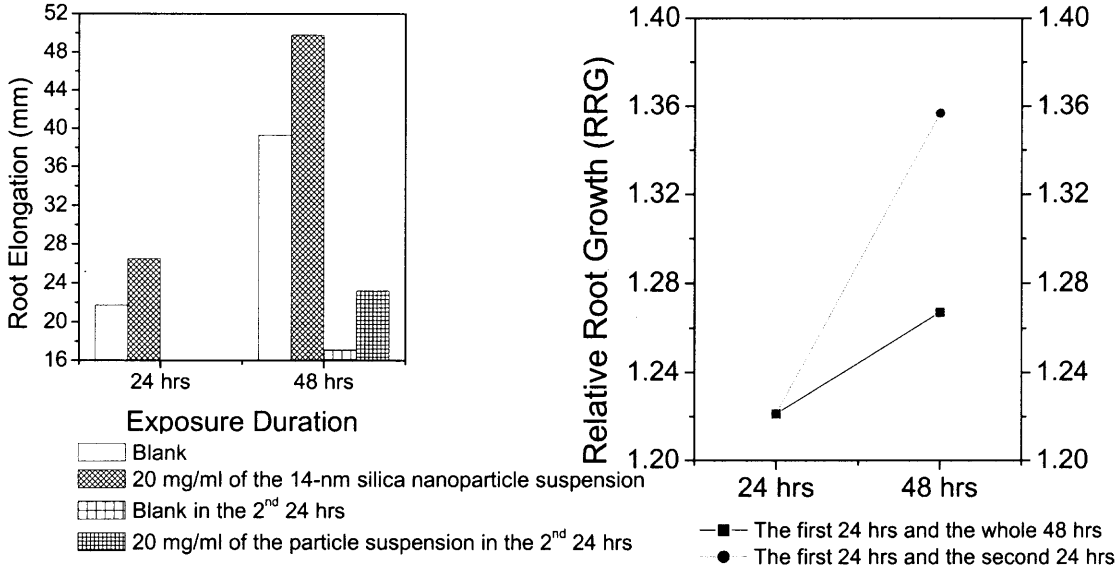


Figure 4.5 Root elongation (RE) and relative root growth (RRG) of seedlings exposed to 14-nm hydrophilic silica particles in the dark at 25 ± 1°C for 24 and 48 hrs.

The mean root growth of the seedlings cultured in the particle suspensions during the total 48 hrs was larger than that of the seedlings cultured in the blanks at all times. The statistical analysis found that during the whole 48 hrs of exposure the p value is 0.0001 for the comparison between the RE of the seedlings exposed to 20 mg/ml of the particle suspensions and that of the seedlings in the blank. The 2nd 24 hrs of exposure

resulted in enhanced root growth of 1.36 fold compared to the blank, with p = 0.0001, which is larger than the 1.22 fold to the blank in the 1st 24 hrs of exposure. The R^2 decreased from 0.8326 in the 1st 24 hrs of exposure to 0.6152 in the 2nd 24 hrs of exposure. It is suspected from the increasing of 1.22 fold to 1.36 fold as well as the decreasing R^2 from 0.8326 to 0.6152, that the enhancing effect of the particles increases with the increase of the exposure duration.

No significant different however was found between the RRG results from the 1st 24 hrs and the 2nd 24 hrs of exposure (p = 0.0899), which means the effect of the silica nanoparticles on plant root growth is not statistically different during both halves of the 48 hrs of exposure. Similarly, no significant difference was found between the RRG results from the 1st 24 hrs and the whole 48 hrs of exposure (p = 0.5260).

The enhancement effect of the 14-nm hydrophilic silica particles, from the data analysis, is suspected to be time-dependent, i.e., the effect increases with the length of exposure duration. This postulation has no statistical significance under the experimental conditions in this study.

4.2.3 Water Treatment Test on the 14-nm Hydrophilic Silica Particles

B. oleracea seedlings were exposed to 20 mg/ml of the 14-nm hydrophilic silica particle suspensions. The particles were removed after 24 hrs from the seedling cultures. The seedlings were rinsed with Milli-Q water to remove any loose particles that were on the seedling surface. The rinse was repeated till the effluent was visibly clear to the eye instead of the cloudy as it originally appeared (approximate 500 ml water was required). The seedlings were then transferred to clean Petri dishes with filter papers and 5 ml Milli-Q water in each dish. The RE was obtained after 24 hrs of treatment (Table 4.9).

The root growth of *B. oleracea* seedlings during the 24 hrs of exposure to 20 mg/ml of the silica nanoparticles was enhanced 1.19 fold compared to the blank (p = 0.02). The removal of the particles however did not return the seedlings growth to that of those cultured in the blanks, as seen in the RE values of seedlings during the 2nd 24 hrs of water treatment (Table 4.9): the mean root elongation of seedlings grown in the blank is 14.6 mm, compared to the 20.2 mm of that of seedlings exposed to the particles, with p = 0.0218. No significant difference has been found between the 1st 24 hrs of exposure to 20 mg/ml silica nanoparticle suspensions and the 2nd 24 hrs of water treatment (p = 0.875, R^2 = 0.9996), as well as between the 2nd 24 hrs of exposure to 20 mg/ml silica particle suspensions and the 24 hrs of water treatment without presence of the silica particles (p = 0.8331, R^2 = 0.9992). This result suggests that the enhancement effect of the silica nanoparticles is not reversible under these conditions. The enhancement effect of the 14-nm hydrophilic particles thus may be deemed as a toxic effect caused by the particles, by definition.

Moreover, no statistically significant difference has been found between the RRG result for 48-hr exposure and that for the water treatment test (p = 0.7725 for the whole 48-hr duration, and 0.6683 for the second 24-hr duration), indicating that the water treatment and the 48-hr exposure had similar effects on the root growth.

4.2.4 Conclusions – the Effect of 14-nm Hydrophilic Silica Particles on Root Growth

Hydrophilic silica particles of 14 nm can enhance the root growth of plant seedlings. This enhancement effect is dose-dependent, with EC90 (90% enhancement concentration) = 5.420 mg/ml as determined using the linear regression equation. It is suspected that the

enhancement effect of the particles increases with the increase of exposure duration, although it is not statistically significant. Results from the root growth after the removal of particles show that the effect of the particles is not reversible. The enhancement effect of the particles is suggested to be an adverse effect, and the 14-nm hydrophilic silica particles are toxic to plant seedling root growth.

Table 4.9 Root Elongation (RE) and Relative Root Growth (RRG) of the *B. oleracea* Seedlings Exposed to 14-nm Hydrophilic Silica Particles for 24 hrs in the Dark at 25 ± 1°C, and Treated with Milli-Q Water for 24 hrs in the Dark at 25 ± 1°C

The results are reported as mean ± S.D. and 95% confidence interval. Statistical different is reported when the p smaller than 0.05.

Exposure duration	Sample	RE mm	RRG
24 hrs	Blank	16.7 ± 5.6, 14.7 ~ 18.7	1.00 ± 0.33, 0.88 ~ 1.12
	20 mg/ml	19.9 ± 5.0, 18.1 ~ 21.7	1.19 ± 0.30, 1.08 ~ 1.30
48 hr	Blank	32.1 ± 8.8, 29.0 ~ 35.3	1.00 ± 0.27, 0.89 ~ 1.11
	20 mg/ml	40.0 ± 9.8, 36.5 ~ 43.6	1.24 ± 0.30, 1.14 ~ 1.36
2nd 24 hrs	Blank	14.6 ± 8.8, 11.0 ~ 18.2	1.00 ± 0.60, 0.75 ~ 1.24
	20 mg/ml	20.2 ± 8.2, 17.2 ~ 23.1	1.38 ± 0.56, 1.18 ~ 1.58
24 hr: Blank vs. 20 mg/ml		p_1[a] = 0.023, p_2[b] = 0.0227, R^2 = 0.9137	
48 hr: Blank vs. 20 mg/ml		p_1 = 0.004, p_2 = 0.0038, R^2 = 0.8468	
2nd 24 hr: Blank vs. 20 mg/ml		p_1 = 0.022, p_2 = 0.0218, R^2 = 0.9011	
20 mg/ml, 1st 24 hrs vs. 2nd 24 hrs		p_1 = 0.875, p_2 = 0.8746, R^2 = 0.9996	

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

4.3 21-nm Titania Particles

4.3.1 24-hr Exposure to the 21-nm Titania Particles

The titania particles studied in this dissertation were purchased from Degussa. The individual particle size, according to the MSDS sheet, is 21nm. The aggregate size of the particles, which was determined by a Coulter N4 plus, is 119.5 ± 58.2 nm. The particles

were suspended in Milli-Q water. The acute toxic effect (i.e., 24-hr toxicity) of the particle suspensions at 20 mg/ml, 2 mg/ml, and 200 μg/ml was investigated by the root elongation test using four plant species: *B. oleracea* (cabbage), *C. sativus* (cucumber), *D. carota* (carrot), and *A. sativa* (oat). The concentration of 20 μg/ml was not tested because in a preliminary study that was designed to determine the test concentration range, the RE of seedlings exposed to 20 μg/ml of the particle suspensions was not different than the RE of seedlings exposed to either 2 mg/ml or 200 μg/ml of particle suspensions. 20 μg/ml was then excluded to facilitate the testing procedure. The RE and RRG during particle exposure was calculated in the testing of 24-hr exposure (Table 4.10) and statistically analyzed (Table 4.11).

Table 4.10 The Root Elongation (RE) and Relative Root Growth (RRG) of Plant Seedlings Exposed to 21-nm Titania Nanoparticles for 24 hrs in the Dark at 25 ± 1°C

The results are reported as mean ± S.D. and 95% confidence interval.

	Concentration	*B. oleracea*	*C. sativus*	*D. carota*	*A. sativa*
RE (mm)	Blank	14.0 ± 4.3 12.3 ~ 15.6	38.4 ± 4.6 37.0 ~ 39.8	8.2 ± 1.5 7.5 ~ 8.9	13.2 ± 4.3 11.4 ~ 15.0
	20 mg/ml	13.2 ± 3.4 11.9 ~ 14.5	35.6 ± 7.5 32.9 ~ 38.2	7.4 ± 1.8 6.5 ~ 8.2	12.6 ± 4.0 11.1 ~ 14.0
	2 mg/ml	16.2 ± 3.9 14.7 ~ 17.6	37.4 ± 4.6 35.7 ~ 39.0	7.4 ± 2.3 6.3 ~ 8.6	13.0± 3.9 11.5 ~ 14.6
	200 μg/ml	14.8 ± 3.4 13.5 ~ 16.2	38.2 ± 6.0 36.0 ~ 40.3	9.2 ± 1.8 8.4 ~ 10.2	13.4 ± 3.9 11.8 ~ 15.0
RRG	20 mg/ml	0.94 ± 0.24 0.85~1.03	0.92 ± 0.19 0.86~0.99	0.90 ± 0.22 0.79 ~ 1.01	0.95 ± 0.30 0.84 ~ 1.06
	2 mg/ml	1.16 ± 0.28 1.05~1.26	0.97 ± 0.12 0.92~1.02	0.91 ± 0.29 0.77 ~ 1.05	0.98 ± 0.30 0.87 ~ 1.10
	200 μg/ml	1.06 ± 0.25 0.96~1.16	0.99 ± 0.16 0.94~1.05	1.13 ± 0.22 1.02 ~ 1.24	1.01 ± 0.29 0.89 ~ 1.13

Ultrafine Titania particles (particles with diameter smaller than 100 nm) have been found more likely to induce the development of particle-mediated lung diseases than the same mass of larger particles (Donaldson et al., 1998, and 1999). They can impair macrophage phagocytosis (Renwick et al., 2001). This study of 24-hr phytotoxicity of titania nanoparticles however showed no difference in root growth of seedlings cultured in the Milli-Q water or grown in the particle suspensions (see Table 4.11).

The average RRG of seedlings grown in the 20 mg/ml, 2 mg/ml, or 200 μg/ml of titania nanoparticle suspensions is 0.93 ± 0.02 (0.91 ~ 0.95), 0.96 ± 0.04 (0.91 ~ 1.00), and 1.04 ± 0.07 (0.97 ~ 1.11), respectively. No significant difference was found between the RRG results of blank (1.00 ± 0.002, 1.00 ~ 1.002) and 2 mg/ml or 200 μg/ml, with p=0.309, and 0.2657. Significant difference with p = 0.0427 exists however between the 20 mg/ml exposure and the blank when the RRG values of the four plant species are combined.

4.3.2 48-hr Exposure to the 21-nm Titania Particles

The 48 hrs of exposure was done with the seedlings of the *C. sativus* at 20 mg/ml and 2 mg/ml of the titania nanoparticle suspensions. The mean value of RE of seedlings after 48 hrs was obtained (Table 4.12).

The root elongation of seedlings exposed to the particles was similar to that of the seedlings cultured in the Milli-Q water during the 1st 24 hrs (p = 0.0506 for 20 mg/ml, 0.3389 for 2 mg/ml), 2nd 24 hrs (p = 0.5831 for 20 mg/ml, 0.3707 for 2 mg/ml), or 48 hrs (p = 0.7508 for 20 mg/ml, 0.1267 for 2 mg/ml). The results indicate that no adverse effect of the titania nanoparticles during the 48 hrs of exposure.

Table 4.11 Statistical Analysis Results of the RE and RRG of Plant Seedlings Exposed to Titania Nanoparticles for 24 hrs in the Dark at 25 ± 1°C

The results in the single concentration and plant species as well as between concentrations are from the analysis between the blank and the concentration, and are based on the RE values. Comparisons between plant species are based on the RRG results. Statistical difference is reported as $p < 0.05$.

	B. oleracea	*C. sativus*	*D. carota*	*A. sativa*
20 mg/ml	p_1[a]=0.447, p_2[b] = 0.4472 R^2 = 0.9888	p_1 = 0.051, p_2 = 0.0506 R^2 = 0.9450	p_1 = 0.150, p_2 = 0.1495 R^2 = 0.9362	p_1 = 0.588, p_2 = 0.5882 R^2 = 0.9941
2 mg/ml	p_1 = 0.058, p_2 = 0.0585 R^2 = 0.9316	p_1 = 0.339, p_2 = 0.3389 R^2 = 0.9865	p_1 = 0.267, p_2 = 0.2670 R^2 = 0.9626	p_1 = 0.894, p_2 = 0.8942 R^2 = 0.9996
200 µg/ml	p_1 = 0.445, p_2 = 0.4446 R^2 = 0.9878	p_1 = 0.833, p_2 = 0.8327 R^2 = 0.9993	p_1 = 0.079, p_2 = 0.0792 R^2=0.9039	p_1 = 0.877, p_2 = 0.8774 R^2=0.9994
20 mg/ml vs. 2 mg/ml	p_1 = 0.004, p_2 = 0.00039 R^2 = 0.9248	20 mg/ml – 2 mg/ml – 200 µg/ml	p_1 = 0.896, p_2 = 0.8960 R^2 = 0.9994	20 mg/ml – 2 mg/ml – 200 µg/ml
20 mg/ml vs. 200 µg/ml	p_1 = 0.089, p_2 = 0.0891 R^2 = 0.9433	p_2 = 0.2492	p_1 = 0.006, p_2 = 0.0065 R^2 = 0.7694	p_2 = 0.7566
2 mg/ml vs. 200 µg/ml	p_1 = 0.207, p_2 = 0.2074 R^2 = 0.9678		p_1 = 0.022, p_2 = 0.0218 R^2=0.8358	
20 mg/ml	All plant species: f = 0.1713, p = 0.9209, no significant difference			
2 mg/ml	The result from the test using the *B. oleracea* is found to be significant different than that from the test using the other three plant species. The other three species: *C. sativus – D.carota – A. sativa*: f = 0.5470, p = 0.5812, no significant difference			
200 µg/ml	All plant species: f = 1.7607, p = 0.2806, no significant difference			

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

Table 4.12 RE and RRG of the *C. sativus* Seedlings after 48 hrs of Exposure to 2 mg/ml and 20 mg/ml of 21-nm Titania Particle Suspensions

The results reported represent the mean value ± S.D. Statistical analysis was performed by the *one-way ANOVA* procedure. Significant difference is indicated as p smaller than 0.05.

		1st 24 hrs	48 hrs	2nd 24 hrs	1st 24 hrs vs. 2nd 24 hrs	1st 24 hrs vs. 48 hrs
RE (mm)	Blank	38.4 ± 4.6 37.0 ~ 39.8	75.2 ± 12.1 71.4 ~ 79.0	36.7± 9.7 33.6 ~ 39.7	p = 0.3107 R^2 = 0.9865	-
	20 mg/ml	35.6 ± 7.5 32.9 ~ 38.2 p = 0.0506 R^2 = 0.9450	76.1 ± 11.0 72.1 ~ 80.1 p = 0.7508 R^2 = 0.9985	35.5 ± 6.6 33.1 ~ 37.9 p = 0.5831 R^2 = 0.9954	p = 0.9891 R^2 = 1.0000	-
	2 mg/ml	37.4 ± 4.6 35.7 ~ 39.0 p = 0.3389 R^2 = 0.9865	69.7 ± 6.4 63.2 ~ 76.1 p = 0.1267 R^2 = 0.9639	34.4 ± 10.8 30.3 ~ 38.5 p = 0.3707 R^2 = 0.9874	p = 0.1983 R^2 = 0.9682	-
RRG	20 mg/ml	0.92 ± 0.19 0.86~0.99	1.01 ± 0.15 0.96~1.06	0.97 ± 0.18 0.90 ~ 1.03	-	p = 0.0577 R^2 = 0.9382
	2 mg/ml	0.97 ± 0.12 0.92~1.02	0.93 ± 0.23 0.84~1.01	0.94 ± 0.29 0.83 ~ 1.05	-	p = 0.3387 R^2 = 0.9833

4.3.3 Conclusions – the Effect of 21-nm Titania Particles on Root Growth

No adverse effect was found with the titania particles on plant seedling root growth.

4.4 161.2-nm Hydrophilic Spherical Silica Particles

4.4.1 24-hr Exposure to the 161.2-nm Hydrophilic Spherical Silica Particles

Three plant species, including *B. oleracea* (cabbage), *C. sativus* (cucumber), and *A. sativa* (oat), were used to investigate the phytotoxicity of the hydrophilic spherical silica particles, of which the size determined by a Coulter N4+ is 161.2 ± 45.6nm. The seedlings were exposed to the particle suspensions at the concentrations of 20 mg/ml, 2

mg/ml and 200 μg/ml. The RE and RRG values during the 24 hrs of exposure is given in Table 4.13.

The difference is significant in the mean RE of the seedlings exposed to the 20 mg/ml titania particle suspensions compared to that of the seedlings cultured in blanks for all of the tests with different plant species. The mean RE of the seedlings when grown in the 20 mg/ml particle suspensions is 0.62 to 0.77 fold of the mean RE of the seedlings in the Milli-Q water, with similar statistical analysis results of p values 0.000 for all of the plant species. The situation for 2 mg/ml was different. The mean RE of the seedlings in the 2 mg/ml of particle suspensions is determined to be significantly different than that of the seedlings in the blank when the plant species of *C. sativus* and *A. sativa* were used, whereas in the test using the *B. oleracea*, no difference can be found, although the mean RRG values of the seedlings of the three plant species are very similar: 0.90 for *B. oleracea*, 0.91 for *C. sativus*, and 0.90 for *A. sativa*. Further statistical analysis was made in order to evaluate the phytotoxicity of the particles (Table 4.14).

The result for *C. sativus* was significantly different from those for the other two plant species *B. oleracea* and *A. sativa* for the 20 mg/ml concentration. The mean RRG at 20 mg/ml was thus calculated from the other two species, resulting in 0.64 ± 0.03 (95% CL is 0.60 ~ 0.69). This mean value was found to be significantly different than the RRG from the blank, with the p 0.0001.

The mean RRG for 2 mg/ml was calculated from the tests using the three plant species because no significant difference was found among the three RRG values for the three plant species, resulting in 0.90 ± 0.01 (0.89 ~ 0.91), which is significantly different than the mean RRG of the seedlings grown in the blank, with the p 0.0012.

Table 4.13 The RE and RRG of Plant Seedlings Exposed to 20 mg/ml, 2 mg/ml, and 200 μg/ml of 161.2-nm Hydrophilic Spherical Silica Particle Suspensions

The results reported are the mean value ± S.D. Statistical analysis was performed by the *Student's t- test* and the *one-way ANOVA* procedure to determine the difference between the RE results from the blanks and the particle suspensions. Significant difference is reported when $p < 0.05$.

		B. oleracea	*C. sativus*	*A. sativa*
RE (mm)	Blank	21.7 ± 5.8, 19.6 ~ 23.9	31.9 ± 5.2, 30.0 ~ 33.7	25.9± 4.1, 24.7 ~ 27.1
	20 mg/ml	13.5 ± 4.1, 11.8 ~ 15.1 p_1[a] = 0.000, p_2[b] = 0.0001, R^2 = 0.5879	24.4 ± 4.4, 22.8 ~ 26.0 p_1 = 0.000, p_2 = 0.0001, R^2 = 0.6182	17.3 ± 5.6, 15.1 ~ 19.5 p_1 = 0.000, p_2 = 0.0001, R^2 = 0.5548
	2 mg/ml	19.5 ± 6.6, 17.2 ~ 21.9 p_1 = 0.184, p_2 = 0.1843, R^2 = 0.9682	29.1 ± 5.1, 27.3 ~ 30.9 p_1 = 0.041, p_2 = 0.0406, R^2 = 0.9296	23.3 ± 4.0, 21.9 ~ 24.8 p_1 = 0.009, p_2 = 0.0085, R^2 = 0.9090
	200 μg/ml	23.0 ± 5.9, 20.7 ~ 25.2 p_1 = 0.450, p_2 = 0.4499, R^2 = 0.9890	31.2 ± 4.3, 29.6 ~ 32.7 p_1 = 0.561, p_2 = 0.5606, R^2 = 0.9940	24.6 ± 2.8, 23.6 ~ 25.6 p_1 = 0.143, p_2 = 0.1428, R^2 = 0.9708
RRG	20 mg/ml	0.62 ± 0.19, 0.54~0.69	0.77 ± 0.14, 0.72~0.82	0.67 ± 0.21, 0.58 ~ 0.75
	2 mg/ml	0.90 ± 0.30, 0.79~1.01	0.91 ± 0.16, 0.86~0.97	0.90 ± 0.16, 0.84 ~ 0.96
	200 μg/ml	1.06 ± 0.27, 0.95~1.16	0.98 ± 0.13, 0.93~1.03	0.95 ± 0.11, 0.91 ~ 0.99

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

Table 4.14 Statistical Analysis Results of the RE and RRG of Plant Seedlings Exposed to 161.2-nm Hydrophilic Spherical Silica for 24 hrs in the Dark at 25 ± 1°C

The difference between the concentrations is evaluated by RE. And the difference between the plant species is evaluated by RRG. Significant difference is reported when the p smaller than 0.05.

	B. oleracea	*C. sativus*	*A. sativa*
20 mg/ml vs. 2 mg/ml	p_1[a] = 0.000, p_2[b] = 0.0001 R^2 = 0.7701	p_1 = 0.000, p_2 = 0.0004 R^2 = 0.8020	p_1 = 0.000, p_2 = 0.0001 R^2 = 0.7111
20 mg/ml vs. 200 μg/ml	p_1 = 0.000, p_2 = 0.0001 R^2 = 0.5301	p_1 = 0.000, p_2 = 0.0001 R^2 = 0.6181	p_1 = 0.000, p_2 = 0.0001 R^2 = 0.5673
2 mg/ml vs. 200 μg/ml	p_1 = 0.045, p_2 = 0.0448 R^2 = 0.9327	p_1 = 0.100, p_2 = 0.0999 R^2 = 0.9532	p_1 = 0.142, p_2 = 0.1424 R^2 = 0.9633
20 mg/ml	Result from the *C. sativus* is significantly different than the results from the other two plant species. For the other two species: p_2 = 0.4061		
2 mg/ml	Three species: p_2 = 0.9575		
200 μg/ml	Three species: p_2 = 0.0844		

[a] Calculated from the *Student's t-test*
[b] Calculated from the *One-way ANOVA* procedure

The mean RRG for 200 μg/ml was also calculated from the tests using the three plant species and is 0.99 ± 0.05 (0.93 ~ 1.06), which was not found to be significantly different than the mean RRG of the seedlings in the blank, with the p 0.8451.

The statistical analysis results performed between the mean RRG for particle suspensions and that for the blank suggest that at either 20 mg/ml or 2 mg/ml, the 161.2-nm hydrophilic spherical silica particles can inhibit the root growth.

A linear regression was evaluated by comparing the logarithm of particle suspension concentrations ($\log_{10}C$) to RRG to determine whether the inhibition effect of the particles on the root growth is dose-dependent. The result is shown in Figure 4.6.

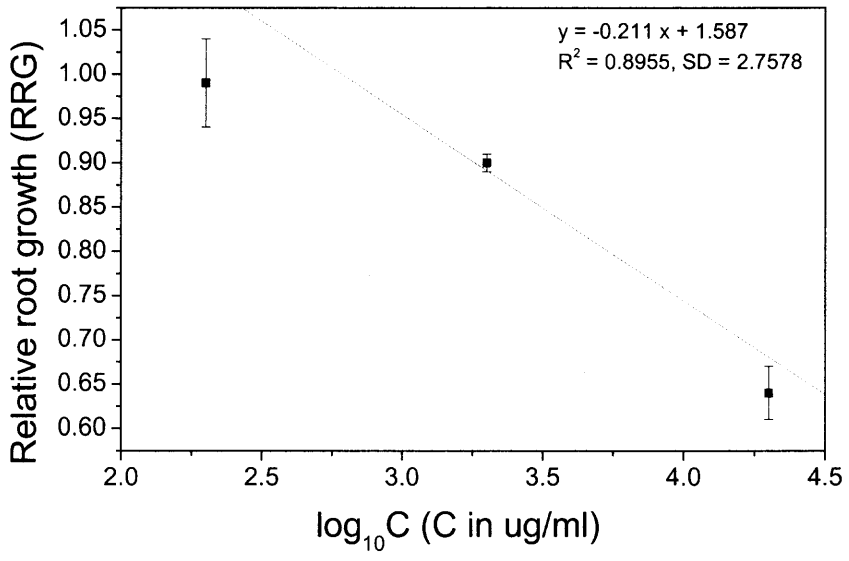


Figure 4.6 Relative root growth of seedlings exposed to 161.2 nm hydrophilic spherical silica particles for 24 hrs in the dark at 25 ± 1°C – mean value of the RRG of seedlings of three plant species. Linear regression evaluated by logarithm of particle suspension concentrations.

Similar to the 13-nm alumina particles, the 161.2-nm hydrophilic silica particles inhibit the root growth of plant seedlings in a dose-dependent manner, with the R^2 0.8955, which means the linear fit for the data analysis is feasible, the RRG is closely related to the logarithm of particle suspension concentration, and the inhibition effect of the particles on the plant root growth is dose–dependent. The particles have no effect on root growth at 200 μg/ml, with p larger than 0.05 (p = 0.0844), which suggests the existence of a phytotoxicity threshold value for 68.1-nm hydrophilic silica particles. The IC_{10} of the particles is about 1.803 mg/ml.

4.4.2 48-hr Exposure to the 161.2-nm Hydrophilic Spherical Silica Particles

The *B. oleracea* seedlings were used to investigate the 48-hr exposure effect of the 161.2-nm hydrophilic spherical silica particles at 20 mg/ml. Figure 4.7 presents the RE results after the first 24 hrs, the 48 hrs, and the second 24 hrs of exposure. The 20 mg/ml

concentration was chosen because the inhibition effect of the particles is the most obvious at this concentration. The average RE ± Standard Deviation (S.D.) during the first 24 hrs of exposure of the seedlings was 14.5 ± 6.6 mm, compared to the 21.7 ± 5.8 mm of the seedlings in the blanks (p = 0.0001, and R^2 = 0.6132). The average RE during the second 24 hrs of exposure of the seedlings was 11.5 ± 5.9 mm, compared to the 17.1 ± 5.0 mm in the blanks (p = 0.0016, and R^2 = 0.7520). All together, the 48 hrs of exposure resulted in the RE of 28.3 ± 9.5 mm of the seedlings in the 20 mg/ml of the particle suspensions, compared to the 39.3 ± 7.3 mm in the blanks.

Statistical analysis for the first 24 hrs and the second 24 hrs as well as the 48 hrs of exposures did not indicate a significant difference in the root growth (p = 0.5157 for the first 24 hrs vs. the total 48 hrs based on the RRG values, and 0.0942 for the first 24 hrs vs. the second 24 hrs based on the RE values).

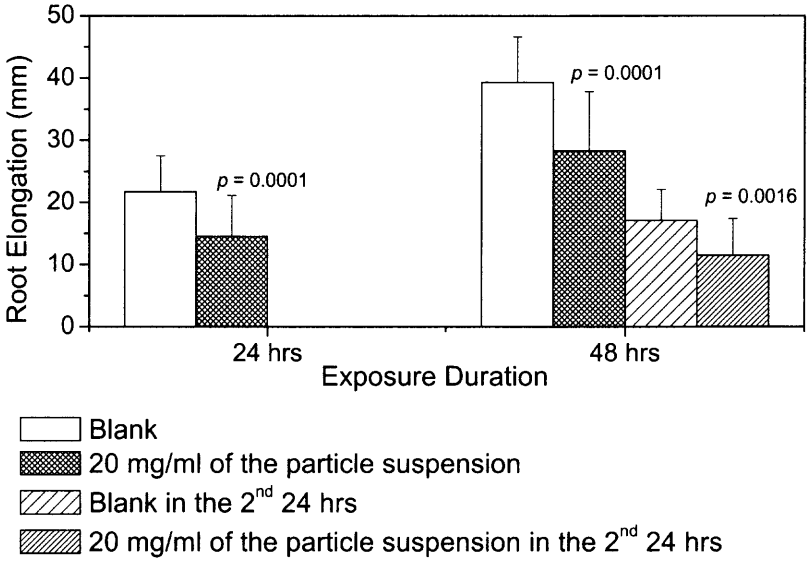


Figure 4.7 Root elongation of seedlings exposed to 161.2-nm hydrophilic spherical silica particles for 24 and 48 hrs in the dark at 25 ± 1°C – compared to the blank.

The root inhibition effect of the 161.2-nm silica particles during the second 24 hrs was less than that of particles during the first 24 hrs because the R^2 between the samples and the blank is larger during the second 24 hrs (0.7520) than during the first 24 hrs (0.6132), although it is not statistically significant. If the inhibition effect of the particles is time-dependent, the RRG at the end of the 48 hrs should be significantly smaller than that at the end of the first 24 hrs. The RRG in this test was 0.67 at the end of the first 24 hrs, and 0.72 at the end of the 48 hrs. It can thus be postulated that the inhibition effect of the particles is not time-dependent in the 48 hrs under the experimental conditions in this study.

4.4.3 Water Treatment Test on the 161.2-nm Hydrophilic Spherical Silica Particles

The experiment of water treatment was designed to evaluate whether the effect of the particles is reversible after the particles have been removed from the seedling cultures. The *B. oleracea* seedlings were first exposed to the 20 mg/ml of the 161.2 nm silica particle suspensions for 24 hrs in the dark at 25 ± 1 °C. The seedlings were removed from the 20-mg/ml particle suspensions at the end of the 24 hrs, and rinsed with Milli-Q water till the effluent was eye-visibly clear instead of the original cloudy. The seedlings were then transferred to clean Petri dishes with filter papers and 5 ml of Milli-Q water in each dish. They were incubated for another 24 hrs in the dark at 25 ± 1 °C. The RE values of the roots were obtained and statistically analyzed (see Table 4.15).

The root growth of the seedlings during the 24 hrs of water treatment in the 20-mg/ml particle suspensions was still slower than that of the seedlings in the Milli-Q water ($p = 0.0036$). There is no difference between the RE of the seedlings in the first 24 hrs of exposure to the particles and the second 24 hrs of water treatment ($p = 0.1614$). It can be

suggested based on this fact, that the inhibition effect of the particles is not reversible under these conditions.

No statistically significant difference has been found between the RRG result for the water treatment test and that for the 48-hr exposure ($p = 0.1235$ for the whole 48 hrs of duration, and 0.8356 for the second 24 hrs of treatment or exposure), indicating that

Table 4.15 The RE, RRG and Statistical Analysis Results of the *B. oleracea* Seedlings Exposed to 161.2-nm Hydrophilic Spherical Silica and then Treated with Water for 24 hrs in the Dark at 25 ± 1°C

The difference between the first 24 hrs of exposure and the second 24 hrs of treatment is evaluated by RRG. All other difference evaluations are based on the RE values. Significant difference is reported when the p is smaller than 0.05.

Exposure duration	Sample	RE mm	RRG
24 hrs	Blank	21.7 ± 5.8, 19.6 ~ 23.9	1.00 ± 0.26, 0.90 ~ 1.10
	20 mg/ml	13.7 ± 2.6, 12.7 ~ 14.7	0.63 ± 0.12, 0.58 ~ 0.67
48 hr	Blank	39.3 ± 7.3, 36.4 ~ 42.3	1.00 ± 0.18, 0.92 ~ 1.07
	20 mg/ml	25.1 ± 8.2, 21.8 ~ 28.5	0.64 ± 0.21, 0.55 ~ 0.72
2nd 24 hrs for water treatment	Blank	17.1 ± 5.0, 15.1 ~ 19.1	1.00 ± 0.29, 0.88 ~ 1.12
	20 mg/ml	11.4 ± 7.6, 8.3 ~ 14.5	0.66 ± 0.44, 0.48 ~ 0.84
24 hr: Blank vs. 20 mg/ml		p_1[a] = 0.000, p_2[b] = 0.0001, $R^2 = 0.5466$	
48 hr: Blank vs. 20 mg/ml		$p_1 = 0.000, p_2 = 0.0001, R^2 = 0.5333$	
2nd 24 hr for water treatment: Blank vs. 20 mg/ml		$p_1 = 0.004, p_2 = 0.0036, R^2 = 0.7848$	
20 mg/ml, 1st 24 hrs vs. 2nd 24 hrs		$p_1 = 0.161, p_2 = 0.1614, R^2 = 0.9569$	

[a] Calculated from the *Student's t-test*
[b] Calculated from the *One-way ANOVA* procedure

the water treatment and the 48-hr exposure had similar effect on root growth. The water treatment did not reverse the inhibition of the particle suspensions of the 161.2-nm silica particles.

4.4.4 Conclusions – the Effect of the 161.2-nm Hydrophilic Spherical Silica Particles on Root Growth

The particles can inhibit the growth of the plant seedlings at 20 mg/ml and 2 mg/ml. There is a threshold value of the toxicity because of the fact that no adverse effect was found for the 161.2-nm silica particles at 200 μg/ml. The IC_{10} (concentration that causes 10% of inhibitory the effect, i.e., RRG = 0.90 in this case) was determined as 1.803 mg/ml according to the linear regression equation. The inhibition effect of the particles is dose-dependent. The largest effect occurs during the first 24 hours, and there is no difference in root growth after the first 24 hours of exposure. The effect is adverse since it is irreversible.

4.5 Sub-micron and Micron Sized Particles

Particles within the same material category (i.e., particles made up of the same chemical material) yet with different sizes were studied in this dissertation (Table 3.1). This section focus on studies of the effects of sub-micron and micron sized particles on the plant seedling growth. The sub-micron and micron sized particles studied include 667.6 nm hydrophilic spherical silica, 0.96 μm titania, and 1.0 μm alumina. Their effects on plant growth were investigated with two plant species: the *B. oleracea*, and the *C. sativus*.

The plant seedlings were incubated with 20 mg/ml and 2 mg/ml of the particle suspensions in the dark at 25 ± 1 °C for 24 hrs. Table 4.16 gives the RE and RRG results.

Table 4.16 The RE and the RRG of Plant Seedlings Exposed to Sub-micron and Micron Sized Particles in the Dark at 25 ± 1°C for 24 hrs

The results are reported as mean ± S.D. and 95% confidence interval. The values of p were calculated from the comparison between particle exposure and negative control. Statistically significant difference is reported when $P < 0.05$.

Plant species	Concentration	667.6 nm SiO_2	0.96 μm TiO_2	1.0 μm Al_2O_3
B. oleracea	Blank	22.4 ± 6.2, 20.1 ~ 24.6	22.4 ± 6.2, 20.1 ~ 24.6	15.6 ± 4.0, 14.1 ~ 17.0
		1.00 ± 0.28, 0.91 ~ 1.09	1.00 ± 0.28, 0.91 ~ 1.09	1.00 ± 0.26, 0.90 ~ 1.09
	20 mg/ml	21.9 ± 7.9, 19.0 ~ 24.7	20.1 ± 4.6, 18.5 ~ 21.8	14.9 ± 4.0, 13.4 ~ 16.4
		0.98 ± 0.35, 0.86~1.09	0.90 ± 0.21, 0.81~0.99	0.95 ± 0.26, 0.85~1.05
		p_1[a] = 0.786,	p_1 = 0.117	p_1 = 0.538
		p_2[b] = 0.7864, R^2 = 0.9987	p_2 = 0.1170, R^2 = 0.9575	p_2 = 0.5383, R^2 = 0.9931
	2 mg/ml	21.4 ± 4.9, 19.6 ~ 23.2	22.9 ± 5.7, 20.9 ~ 25.0	15.3 ± 4.3, 13.7 ~ 17.0
		0.96 ± 0.22, 0.86~1.05	1.02 ± 0.26, 0.93~1.12	0.98 ± 0.28, 0.88~1.09
		p_1 = 0.505	p_1 = 0.726	p_1 = 0.815
		p_2 = 0.5051, R^2 = 0.9922	p_2 = 0.7265, R^2 = 0.9978	p_2 = 0.8149, R^2 = 0.9990
C. sativus	Blank	33.0 ± 4.1, 31.6 ~ 34.5	33.0 ± 4.1, 31.6 ~ 34.5	33.0 ± 4.1, 31.6 ~ 34.5
		1.00 ± 0.12, 0.94 ~ 1.06	1.00 ± 0.12, 0.94 ~ 1.06	1.00 ± 0.12, 0.94 ~ 1.06
	20 mg/ml	33.8 ± 4.0, 32.3 ~ 35.2	32.2 ± 5.4, 30.2 ~ 34.1	32.3 ± 3.1, 31.1 ~ 33.4
		0.96 ± 0.12, 0.92~1.01	0.91 ± 0.16, 0.86~0.97	0.98 ± 0.094, 0.94 ~ 1.02
		p_1 = 0.491	p_1 = 0.484	p_1 = 0.424
		p_2 = 0.4907, R^2 = 0.9918	p_2 = 0.4837, R^2 = 0.9915	p_2 = 0.4245, R^2 = 0.9888
	2 mg/ml	31.0 ± 4.6, 29.3 ~ 32.7	33.8 ± 3.1, 32.5 ~ 35.2	32.7 ± 3.4, 31.4 ~ 33.9
		0.94 ± 0.14, 0.89~0.99	0.99 ± 0.10, 0.95~1.03	0.99 ± 0.10, 0.95 ~ 1.03
		p_1 = 0.075	p_1 = 0.477	p_1 = 0.697
		p_2 = 0.0749, R^2 = 0.9456	p_2 = 0.4769, R^2 = 0.9894	p_2 = 0.6971, R^2 = 0.9974

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

The 24 hrs of exposure to all of the three particles did not produce significant change of the root growth even at 20 mg/ml, compared to the blanks, with p from 0.1170 to 0.7864. The RE results from 20 mg/ml and 2 mg/ml are not statistically different: for the 1-μm alumina particles, the p is 0.7268 for the *B. oleracea*, and 0.6541 for the *C. sativus*; for the 0.96-μm titania particles, the p is 0.4060 for the *B. oleracea*, and 0.2243 for the *C. sativus*; and for the 667.6-nm silica particles, the p is 0.7830 for the *B. oleracea*, and 0.1620 for the *C. sativus*.

4.6 Summary and Conclusions

The effect on plant seedling growth of three categories of manufactured particulate materials with multiple sizes was investigated in this chapter by the root elongation test. Phytotoxicity of the particulate materials in this dissertation is interpreted as either an inhibition effect or an enhancement effect of the particles, because either of the two effects results in abnormal seedling growth compared to Milli-Q water used as the control. It was found that either of the two effects caused by the particles is irreversible under the conditions of the experiments. The 13-nm alumina particles in this sense are suggested to be the most phytotoxic, since the IC_{10} of the particles is 0.281 mg/ml as determined by the linear regression equation, compared to the 5.420 mg/ml for the 14-nm hydrophilic spherical silica, and the 1.803 mg/ml for the 161.2-nm hydrophilic spherical silica particles. The 21-nm titania particles were not found to have any impact on the root growth, thus suggesting that they have no toxic effects on the plant seedling growth.

The 48-hr or 72-hr exposure experiments were designed to investigate if the effect on the root growth of the particles is time-dependent. A 72-hr exposure study was

performed for the 13-nm alumina particles. The study found that the relation (R^2) of the RE of seedlings in the alumina particle suspensions relative to the blank controls was the smallest during the first 24 hrs of exposure (0.8841 for *C. sativus* at 24 hrs of exposure, and 0.6224 for *B. oleracea* at 24 hrs of exposure), and increased with the exposure duration (increased to 0.9870 and 0.9983 for *C. sativus* at the second 24 hrs and the third 24 hrs of exposure respectively, and to 0.8153 for *B. oleracea* at the second 24 hrs of exposure). This result indicates that the largest inhibition effect of the 13-nm alumina occurs during the first 24 hrs of exposure under the experimental conditions. The results from the 48-hr exposure to the 161.2-nm hydrophilic spherical silica particles suggest the same thing that the largest inhibitory effect of the particles occurs during the first 24 hrs of exposure.

The situation is different for the 14-nm hydrophilic spherical silica particles. There is an enhanced growth and there is a larger effect in the 2nd 24-hr period. Less R^2 occurred between the second 24 hrs and the blank control, which means that the enhancement of root growth is larger in the second 24 hrs. The effect on plant growth of the 14-nm hydrophilic spherical silica particles is supposed to be time-dependent, although no significant difference was found between the RRG in the first 24 hrs and the second 24 hrs. The difference of the time-dependence pattern of different particles suggests that there are different toxic effect-inducing mechanisms of the particles.

Generally, nanometer sized particles (13-nm alumina particles, 14-nm hydrophilic particles, and 161.2-nm hydrophilic spherical silica particles) are more toxic to plant seedling growth than the particles with the same chemical composition and larger sizes (1.0-μm alumina particles, and 667.6-nm hydrophilic silica particles). Sub-micron and

micron sized particles including 1.0-μm alumina, 0.96-μm titania, and 667.6-nm silica particles were not found toxic to the plant seedling growth. Particle size alone however is not the deciding factor, because the 21-nm Titania does not have any kind of effect on the seedling growth. The particle properties that may account for the particle toxicity are discussed in Chapter 6.

CHAPTER 5

INFLUENCE OF PHENANTHRENE LOADING ON PARTICLE PHYTOTOXICITY AND THE MECHANISM STUDY

The toxic effect of the manufactured particulate materials has been investigated in Chapter 4. The toxicity of 13-nm alumina, 14-nm silica, and 161.2-nm silica has been determined. The toxic effect of chemical loaded particles is studied in this chapter. The 24-hr exposure protocol is used for the investigation. This study is to investigate the potential influence of chemicals associated with the particle surface on the particle toxicity. Phenanthrene (Phen) is chosen as the study chemical because it is a major component of airborne and particle surface associated polycyclic aromatic hydrocarbons (PAHs).

5.1 Phen-loaded 13-nm Alumina Particles

5.1.1 Loading of the Phenanthrene (Phen)

The amount of Phen used in the loading process was calculated according to Equation 5.1, and given in Table 5.1. Phen was weighed and dissolved in 4 ml of acetone. Samples of particles, 0.5 grams of 13-nm alumina particles were weighed and dispersed by continuous stirring into the 4 ml Phen-acetone solution to make the "loaded" particles. 0.5 grams of 13-nm alumina particles were at the same time weighed and dispersed into 4 ml acetone to make the "nonloaded" particles. The slurries were left under vacuum at 38 ± 1°C overnight, and kept in vacuum for 24 hrs to ensure the removal of residue acetone. The effects on plant seedling growth of the loaded particles and the nonloaded particles

were then tested. The acetone volume of 4 ml was selected based on preliminary studies, which demonstrated that 4 ml of solvent was enough to immerse 0.5 grams of the nanoparticles.

The amount of Phen used in coating was calculated from the molecular cross section area of Phen and the total surface area of the particles. The amount of phenanthrene needed for a 100.0 % monomolecular layer (MML) of loading, is:

$$M = 178.22 \times [(m \times s)/(6.02 \times 10^{23} \times S \times 10^{-20})] \quad (5.1)$$

where, M is the mass of phenanthrene (g), m is the mass of particles (g), s is the particle specific surface area (m^2/g), S is 107.3 $Å^2$, the molecular cross Section area of Phen (Barbas et al., 1996), and 178.22 is the molecular weight of Phen (g/mol).

Table 5.1 gives the amount of Phen loaded.

Table 5.1 The Amount of Phenanthrene used in Chemical Loading for the Study of Influence of Particle Surface Associated Chemicals on Particle Toxicity

0.5 grams of the 13-nm alumina particles were used for Phen-loading, or for make "nonloaded" particles.

	Phen (M.W. 178.22)		
As % MML	10.0 % MML	100.0 % MML	432.4% MML
As milligrams in the 4 ml acetone	1.42 mg	14.20 mg	61.40 mg
As milligram per gram of the 13-nm alumina particles	2.83 mg/g	28.40 mg/g	122.80 mg/g
As mole per gram of the 13-nm alumina particles	1.6×10^{-5} mol/g	1.6×10^{-4} mol/g	6.9×10^{-4} mol/g

It is possible during the loading process that some Phen also evaporated under the condition of vacuum with heat (the vapor pressure of Phen at 40°C is 215 mm Hg). Therefore, the amount of Phen may be actually somewhat less than the monomolecular layers that were presented in Table 5.1. FTIR studies however show that detectable levels of Phen were loaded in all three of the MML Phen loadings (see Section 5.1.2).

Theoretically, the particles were evenly coated. This may not be true in actuality. Some places on the particle surfaces might be loaded with more than a monomolecular layer of Phen, and exceed the level of MML that is reported, while some other surfaces might not be loaded.

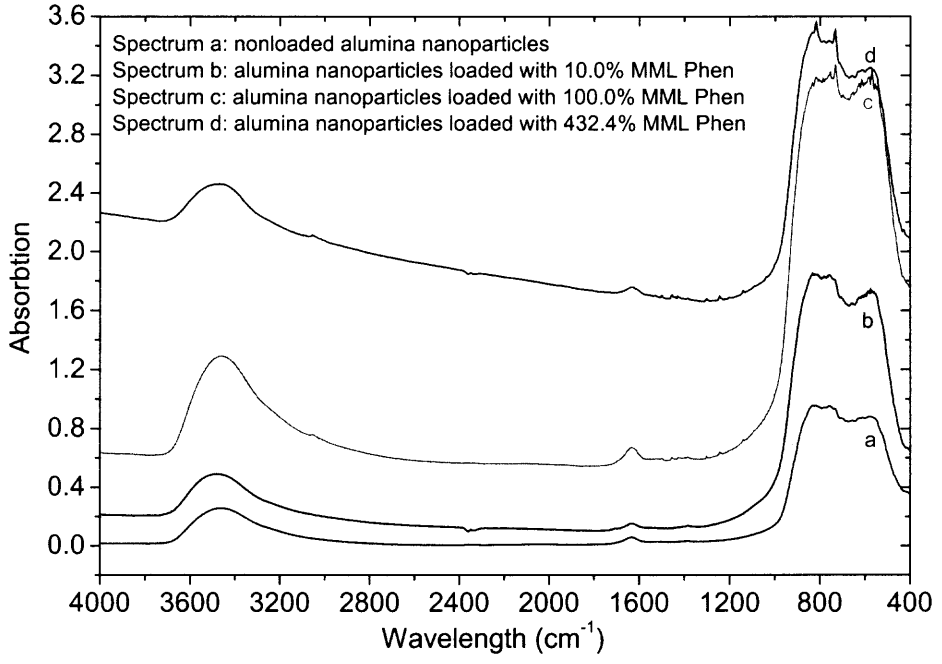


Figure 5.1 FTIR spectra of nonloaded alumina particles (a), particles loaded with 10.0 % MML (b), 100.0 % MML (c), and 432.4 % MML (d) of phenanthrene.

5.1.2 FTIR Study on the Nonloaded and Phen-Loaded Particles

10 mg of particle samples were mixed in 200 mg of ground KBr (IR grade, 99+%, Fisher Scientific). The powder was pressed into pellets (Ø = 10 mm). Each pellet contained 3 to 5 mg of the particles. The instrument recorded 2500 scans on each pellet. The background spectrum was also collected by 2500 scans on a KBr pellet. The spectrometer used was a Mattson Research Series FT-IR.

The spectra of nonloaded particles, along with particles loaded with Phen are given in Figure 5.1 and Figure 5.2.

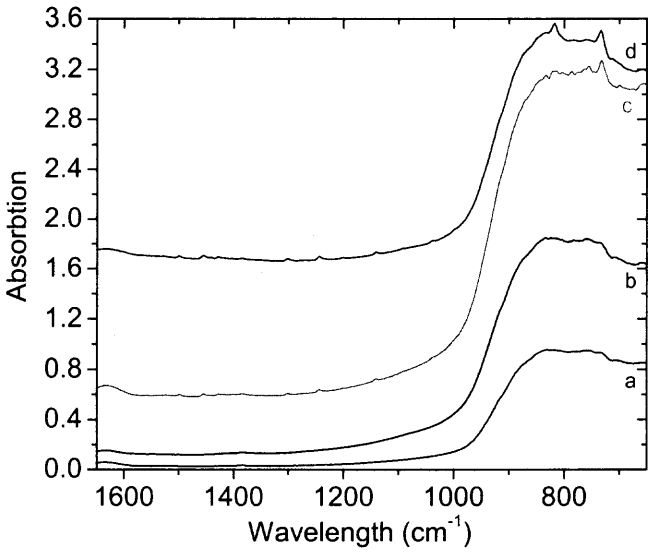
A band in the 850 ~ 1050 cm^{-1} range appears after subtraction of the spectrum of nonloaded particles from the spectra of loaded particles. This band was assigned to surface vibrational modes of alumina nanoparticles and its appearance arises from the disappearance of free alumina hydroxyl (OH) groups (Lavalley and Benaissa, 1985).

5.1.3 Phytotoxicity Study by the Root Elongation Test

The phytotoxicity of the Phen-loaded particles was investigated by the root elongation test using the *C. sativus* seedlings. The seedlings were exposed to 2 mg/ml of the nonloaded particles or the10.0% MML, 100.0% MML, and 432.4% MML of Phen-loaded particles. Table 5.2 and Figure 5.3 present the RE and RRG values. The toxicity of Phen was tested as well at the same level as in the 2 mg/ml of particles loaded with 432.4% ML of Phen (0.28 mg/ml) (see Chapter 3, Section 3.2.3 for the procedure).

The RE values of *C. sativus* seedlings exposed to 2 mg/ml suspensions of 10.0% MML and 100.0% MML of Phen-loaded particles are very similar to those of the seedlings in the blank control (p is 0.341 and 0.632, R^2 is 0.9870 and 0.9960), but it was

(I)



(II)

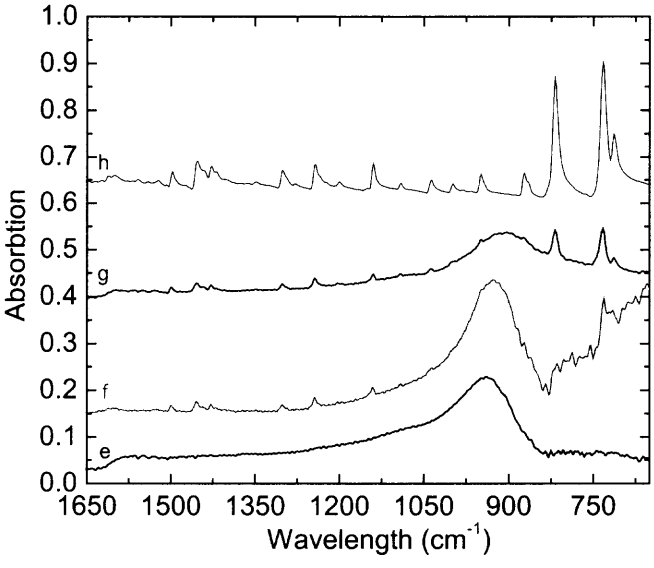


Figure 5.2 FTIR spectra from wavenumber 600 cm^{-1} to wavenumber 1650 cm^{-1}. (I) Nonloaded alumina particles (***a***), particles loaded with 10.0 % MML of phenanthrene (***b***), 100.0 % MML of phenanthrene (*c*), and 432.4 % MML of phenanthrene (***d***); and (II) spectra obtained by subtraction of ***a*** from ***b***, *c*, and ***d***, resulting in ***e***, *f*, and ***g*** respectively. Spectrum ***h*** is of phenanthrene that was obtained from the pellet made of the compound and KBr. The band near 950 cm^{-1} is related to the surface characteristics of alumina nanoparticles (Lavalley and Benaissa, 1985). And the absorbance of Phen can be distinguished in both spectrum *f* and spectrum ***g***.

significantly different than the RE values of the seedlings exposed to the 2 mg/ml of the nonloaded particles (p is 0.000 and 0.0067, R^2 is 0.8200 and 0.8907). Moreover, the RRG values of *C. sativus* seedlings exposed to 2 mg/ml of suspensions of Phen-loaded particles increased to 1.24 fold (10.0%), 1.21 fold (100.0%), and 1.15 fold (432.4%) to those of the seedlings exposed to 2 mg/ml of suspensions of nonloaded particles, with p = 0.0001 (10.0%), 0.0067 (100.0%), and 0.0681 (432.4%), respectively, which means that the toxicity of the particles is reduced significantly compared to the Phen-nonloaded alumina particles. The situation of the 432.4% MML of Phen-loaded particles is complicated. The RE of the seedlings grown in the presence of the 2 mg/ml suspension of the 432.4% MML of Phen-loaded particles was comparable to of the RE of the seedlings grown in the blank control (p = 0.228), at the same time it was comparable to the RE of the seedlings exposed to the 2 mg/ml of the nonloaded particles (p = 0.068). It can however still be stated that the 432.4% MML of Phen-loaded particles are not toxic to the root growth, because whether the sample is toxic or not is concluded referring to the negative control, i.e., the blank in this study.

The three levels of loaded particles have increasing R^2 compared with the nonloaded particles (from 0.8200 for the 10.0% MML of Phen-loaded particles, 0.8907 for the 100.0% MML of Phen-loaded particles, to 0.9489 for the 432.4% MML of Phen-loaded particles). This increase in R^2 indicates that the toxic effect of the loaded particles increases with the loading amount of the Phen, and approaches the inhibitory effect of the nonloaded alumina particles. This phenomenon is assigned to the toxicity of the particle loaded Phen, not the particles, because the only thing that changed in these three particle

Table 5.2 Phen Loading Changes the Phytotoxicity of the 13-nm Alumina Particles

Phytotoxicity was evaluated by root elongation test using the *C. sativus* seedlings, at 2 mg/ml of the particle suspensions, in the dark at 25 ± 1 °C for 24 hrs. Results are expressed as mean ± S.D. and 95% confidence interval.

		Nonloaded	10.0%MML of Phen - loaded	100.0%MML of Phen - loaded	432.4%MML of Phen - loaded	0.28 mg/ml Phen[a]
RE	Blank	27.0 ± 6.5, 24.8 ~ 29.1	27.0 ± 6.5, 24.8 ~ 29.1	20.0 ± 6.0, 17.9 ~ 22.2	20.0 ± 6.0, 17.9 ~ 22.2	24.5 ± 4.8, 22.9 ~ 26.1
	Sample	22.6 ± 3.8, 21.4 ~ 23.8	28.4 ± 5.8, 26.4 ~ 30.3	19.4 ± 4.5, 17.7 ~ 21.0	18.4 ± 4.2, 16.9 ~ 19.9	26.0 ± 6.0, 24.0 ~ 28.0
	(compared to the blank)	p_1[b] = 0.000 p_2[c] = 0.0009 R^2 = 0.8538	p_1 = 0.341 p_2 = 0.3407 R^2 = 0.9870	p_1 = 0.632 p_2 = 0.6321 R^2 = 0.9960	p_1 = 0.228 p_2 = 0.2281 R^2 = 0.9750	p_1 = 0.266 p_2 = 0.2657 R^2 = 0.9821
RRG		0.83 ± 0.14, 0.79~0.88	1.05 ± 0.22, 0.98~1.12	0.97 ± 0.23, 0.88~1.05	0.92 ± 0.21, 0.84~0.99	1.06 ± 0.24, 0.98~1.14
	Compared to the nonloaded particles	N/A	p_1 = 0.000 p_2 = 0.0001 R^2 = 0.8200	p_1 = 0.0067 p_2 = 0.0067 R^2 = 0.8907	p_1 = 0.068 p_2 = 0.0681 R^2 = 0.9489	N/A
	10.0% MML – 100.0% MML	N/A		p_1 = 0.132 p_2 = 0.1320 R^2 = 0.9649		N/A
	10% MML – 432.4% MML			p_1 = 0.015 p_2 = 0.0148 R^2 = 0.9107		
	100.0% MML – 432.4% MML			p_1 = 0.398 p_2 = 0.3983 R^2 = 0.9877		

[a] The same amount of Phen in the 2 mg/ml of the 432.4% MML of Phen-loaded particles
[b] Statistical analysis performed by the *Student's t-test*
[c] Statistical analysis performed by the *one-way ANOVA* procedure.

suspensions was the amount of the Phen that had been loaded. The 0.28 mg/ml of Phen, which is the same amount of Phen as contained in the 2 mg/ml of the 432.4% MML of Phen-loaded particle suspension, when tested separately, was found to be nontoxic to the root growth. The particles loaded with the same amount of the Phen however, were significantly increased in the inhibitory effect on root growth than the Phen alone (p = 0.0165). A possible explanation for the observation is that the bioavailability to the root of Phen may be increased after it becomes attached to the particle surfaces.

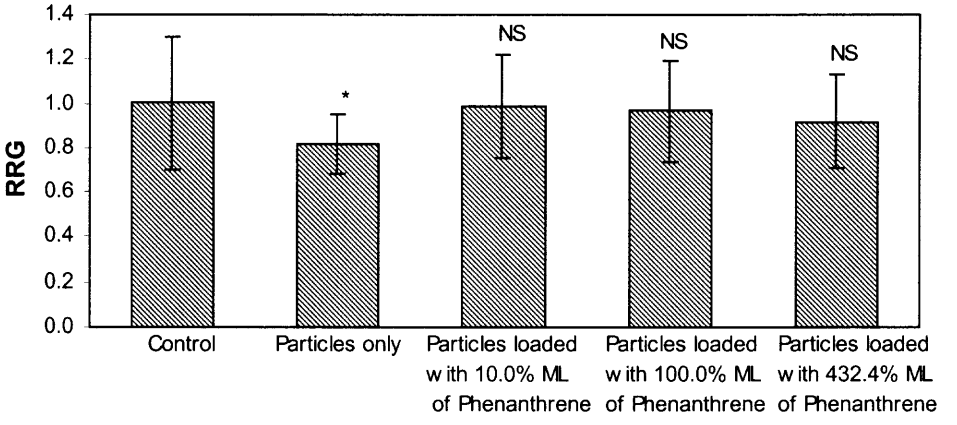


Figure 5.3 The RRG of the *C. sativus* seedlings after their exposure for 24 hrs to 2 mg/ml of nonloaded alumina nanoparticles or 2 mg/ml of alumina nanoparticles loaded with 10.0 %, 100.0 %, and 432.4 % MML of Phen. The values are expressed as mean ± S.D. (NS = not significantly different to control, * = $P < 0.05$, by the *Student's t-test*).

The influence on the toxicity of particles with the 10.0% MML of Phen loading was further investigated using additional four plant species: *Z. mays*, *G. max*, *B. oleracea*, and *D. carota*. Table 5.3 presents the RE and RRG results of the seedlings exposed to the nonloaded particles or 10.0 % MML of Phen– loaded particles. Figure 5.4 shows the RE of the seedlings.

The nonloaded particles in this study were not the original particles. They were instead treated with 4 ml of acetone in the same way as the coating process (see Section 5.1.1) in order to provide a more realistic experimental condition. Results from the phytotoxicity studies found no difference in the toxicity exists between the exposure to the 2 mg/ml original particles and exposure to the same concentration of particles treated with acetone (see the according data in Table 4.1 and Table 5.2).

Table 5.3 Phytotoxicity Change Induced by 10.0% MML of Phen Loading of 13-nm Alumina Particles

Phototoxicity is evaluated by seedling root elongation of *Z. may*, *G. max*, *B. oleracea*, and *D. carota*. Results are reported as mean ± S.D. and 95% confidence interval.

		Z. mays	*G. max*	*B. oleracea*	*D. carota*
	Blank	20.02 ± 6.0	37.6 ± 11.2	18.8 ± 3.8	13.2 ± 1.5
RE		17.9 ~ 22.2	32.7 ~ 42.6	17.3 ~ 20.0	12.6 ~ 13.9
	Nonloaded	16.3 ± 2.7	31.5 ± 7.2	14.8 ± 2.4	11.2 ± 4.1
		15.4 ~ 17.3	28.9 ~ 34.1	14.0 ~ 15.5	9.7 ~ 12.6
		p_1[a] = 0.0033	p_1 = 0.0232	p_1 = 0.000	p_1 = 0.034
		p_2[b] = 0.00327	p_2 = 0.0232	p_2 = 0.0001	p_2 = 0.0337
		R^2 = 0.8604	R^2 = 0.8972	R^2 = 0.7082	R^2 = 0.9076
	10.0% MML	19.8 ± 4.7	36.5 ± 10.6	19.0 ±3.3	13.3 ± 3.6
		18.1 ~ 21.5	32.7 ~ 40.3	17.9 ~ 20.0	12.0 ~ 14.6
		p_1 = 0.877	p_1 = 0.7253	p_1 = 0.84	p_1 = 0.919
		p_2 = 0.8770	p_2 = 0.7253	p_2 = 0.8398	p_2 = 0.9191
		R^2 = 0.9996	R^2 = 0.9974	R^2 = 0.9995	R^2 = 0.9998
	Nonloaded	p_1 = 0.0008	p_1 = 0.03716	p_1 = 0.000	p_1 = 0.033
	vs.	p_2 = 0.0008	p_2 = 0.0372	p_2 = 0.0001	p_2 = 0.0330
	10.0% MML	R^2 = 0.8231	R^2 = 0.9273	R^2 = 0.6419	R^2 = 0.9227
RRG	Nonloaded	0.82 ± 0.14	0.84 ± 0.19	0.78 ± 0.13	0.82 ± 0.30
		0.77 ~ 0.86	0.77 ~ 0.91	0.74 ~ 0.82	0.71 ~ 0.93
			p_2[c] = 0.7621		
	10.0% MML	0.99 ± 0.23	0.97 ± 0.28	1.01 ± 0.17	0.98 ± 0.26
		0.91 ~ 1.07	0.87 ~ 1.07	0.95 ~ 1.06	0.89 ~ 1.08
			p_2[c] = 0.8427		

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure
[c] The results from the *C. sativus* were included

The nonloaded particles can inhibit the root growth in a significant manner: the average p value is about 0.015 when compared to the control. The effect on root growth of the 10.0% MML Phen-loaded particles however is significantly different than the nonloaded particles: the average p value is about 0.018. The loaded particles have no detectable effect on root growth: the average p value is about 0.84 when compared to the control. This result, along with that found in the study discussed above, demonstrates that the Phen-loading reduces the inhibitory effect of the particles significantly.

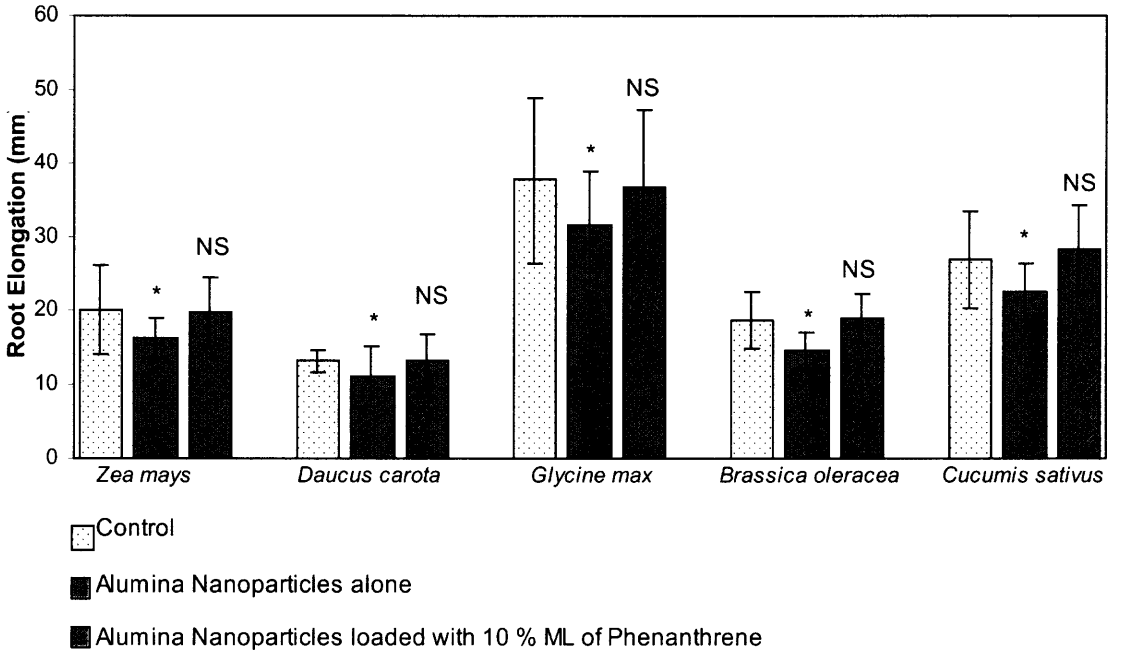


Figure 5.4 The average RE of seedlings after their exposure for 24 hrs to 2 mg/ml of nonloaded alumina nanoparticles or 2 mg/ml of alumina nanoparticles loaded with 10.0 % MML of Phen. The values are expressed as mean ± S.D (NS = not significantly different to control, * = $P < 0.05$, *Student's t-test*).

5.1.4 Study on the Possible Mechanisms

5.1.4.1 Size of the Particles. The size of the alumina nanoparticles might be changed after the Phen-loading, which possibly would induce the change in the toxic effect of the particles. To investigate this possibility, the size of the loaded particles was analyzed by the instrumentation of a Coulter N4+ (Table 5.4).

No significant change within error tolerance in the size has been found. The size of the particles remained almost unchanged after the Phen-loading. The possibility that the change in the particle size induces the change in the toxic effect of the particles does not exist.

Table 5.4 Size of the Nonloaded and Phen-loaded Alumina Nanoparticles

	Original	Nonloaded	10.0% MML Phen – loaded	100.0% MML Phen – loaded	432.4% MML Phen – loaded
Size (nm)	201.0 ± 74.7	217.9 ± 72.1	210.9 ± 87.3	214.0 ± 84.9	225.7 ± 92.0

5.1.4.2 Other Possibilities. The FTIR study on the nonloaded and Phen-loaded alumina particles found that an infrared band in the 850 ~ 1050 cm^{-1} range appears after the particles are loaded with 10.0% MML, 100.0% MML or 432.4% MML of Phen. This band was assigned to surface vibrational modes of alumina nanoparticles and its appearance was suggested to arise from the disappearance of free alumina hydroxyl (·OH) groups (Lavalley and Benaissa, 1985).

The particles were treated with dimethyl sulphoxide (DMSO) in order to

determine whether the free hydroxyl groups are important to the toxic effect of the alumina nanoparticles. DMSO in water can react with •OH, forming stable products (Tai et al., 2002; Zhang et al., 2002).

$$^{\bullet}OH + (CH_3)_2SO \rightarrow CH_3SO(OH) + {}^{\bullet}CH_3 \quad (5.2)$$

$$^{\bullet}CH_3 + O_2 \rightarrow CH_3OO^{\bullet} \quad (5.3)$$

$$2CH_3OO^{\bullet} \rightarrow HCHO + CH_2OH + O_2 \quad (5.4)$$

The alumina particles were dispersed in 5 ml of Milli-Q water at 2 mg/ml, in which 25 µl [0.5% (v/v)] and 50 µl [1.0% (v/v)] of DMSO had been added. The toxicity of the treated particles was then tested by the root growth of *Z. mays* seedlings in triplicates. Table 5.5 presents the results.

The treatment of 0.5% or 1.0 % of DMSO increased the root elongation of seedlings compared to the untreated particles to 1.39-fold (p = 0.0001) or 1.24-fold (p = 0.002) respectively, and was not statistically different than the negative controls (0.5%: p = 0.301 for the blank control, and 0.9111 for the 0.5% DMSO in water that was tested as the solvent control; and 1.0%: p = 0.656 for the blank control, and 0.1252 for the 1.0% DMSO in water that was tested as the solvent control). The result suggests that the addition of DMSO was causing the reduction in OH groups, and significantly reduced the phytotoxicity of the alumina nanoparticles under these conditions.

Table 5.5 The RE and RRG Results of *Z. mays* Seedlings after their Exposure for 24 hrs in the Dark at 25 ± 1°C to 2 mg/ml Alumina Nanoparticles Treated with 0.5% or 1.0% v/v of DMSO

Results are reported as mean ± S.D. along with 95% confidence interval.

	RE	RRG
Blank 1 for DMSO	19.7 ± 9.8, 16.2 ~ 23.2	1.00 ± 0.50, 0.82 ~ 1.18
0.5% DMSO	21.0 ± 7.2, 18.4 ~ 23.6 p_1[a] = 0.542, p_2[b] = 0.5420 R^2 = 0.9936	1.07 ± 0.40, 0.94 ~ 1.20
1.0% DMSO	21.5 ± 6.9, 19.0 ~ 24.0 p_1 = 0.412, p_2 = 0.4124 R^2 = 0.9882	1.09 ± 0.36, 0.96 ~ 1.22
Blank for DMSO – treated and Untreated Particles	17.9 ± 5.4, 16.0 ~ 19.8	1.00 ± 0.30, 0.89 ~ 1.11
Untreated Particles	13.9 ± 3.6, 12.7 ~ 15.2 p_1 = 0.0014, p_2 = 0.001398 R^2 = 0.8374	0.78 ± 0.20, 0.71 ~ 0.85
Particles treated with 0.5% DMSO	19.3 ± 5.1, 17.5 ~ 21.1 p_1 = 0.301, p_2 = 0.3013 R^2 = 0.9816	1.08 ± 0.28, 0.98 ~ 1.18
Particles treated with 1.0% DMSO	17.3 ± 4.4, 15.7 ~ 18.9 p_1 = 0.656, p_2 = 0.6561 R^2 = 0.9966	0.97 ± 0.25, 0.88 ~ 1.06

[a] Analyzed by the *Student's t-test*
[b] Analyzed by the *One-way ANOVA* procedure

5.1.5 SEM Study on the Particle-Exposed Root Samples

The *Z. mays* seedlings were exposed to 5-ml 2 mg/ml of nonloaded or 10.0% MML of Phen-loaded alumina particle suspensions for 24 hrs before the SEM study was initiated. The primary roots of the seedlings were cut, fixed, dehydrated, and dried after exposure. The specimens were sliced from the sites that were about 5 mm from the root tips. The

slices were then sputter-coated with gold, and observed by the SEM (see Chapter 3, Section 3.2.4 for the details of this procedure). The EDS was used to collect the energy of X-ray emitted by element Al at accelerating voltage of 5 keV and magnification of 5 kX. The collection time for the EDS spectra was 20 minutes.

The results from the EDS analysis on the element aluminum (Al) demonstrate that fewer particles were inside the plant roots after the particles have been loaded with 10.0% MML of Phen than the nonloaded particles (Figure 5.5).

5.1.6 Summary and Conclusions

The phytotoxic effect of the 13-nm alumina particles after being loaded with Phen has been investigated in this study. The results demonstrate that after the Phen loading, the phytotoxicity of the particles decreased significantly even to a nontoxic level. That alteration is the opposite of what has been found in the study of the toxic effect of particles involving human cells. This non-agreement may come from the difference between the study objects: the particles that have been studied on human cells are not the 13-nm alumina particle, and the study object in this dissertation is the plant seedling root system, whereas the objects in other published studies are human cells.

The FTIR results suggested that after the Phen loading, the particle surface had been changed. It is suggested that during the loading process, the free hydroxyl groups on the particle surface are altered. The further study on the toxicity change of particles treated with 0.5% or 1.0% DMSO may support this proposal. The SEM/EDS analysis of the root samples indicates fewer of the loaded particles inside the roots, compared to the nonloaded particles.

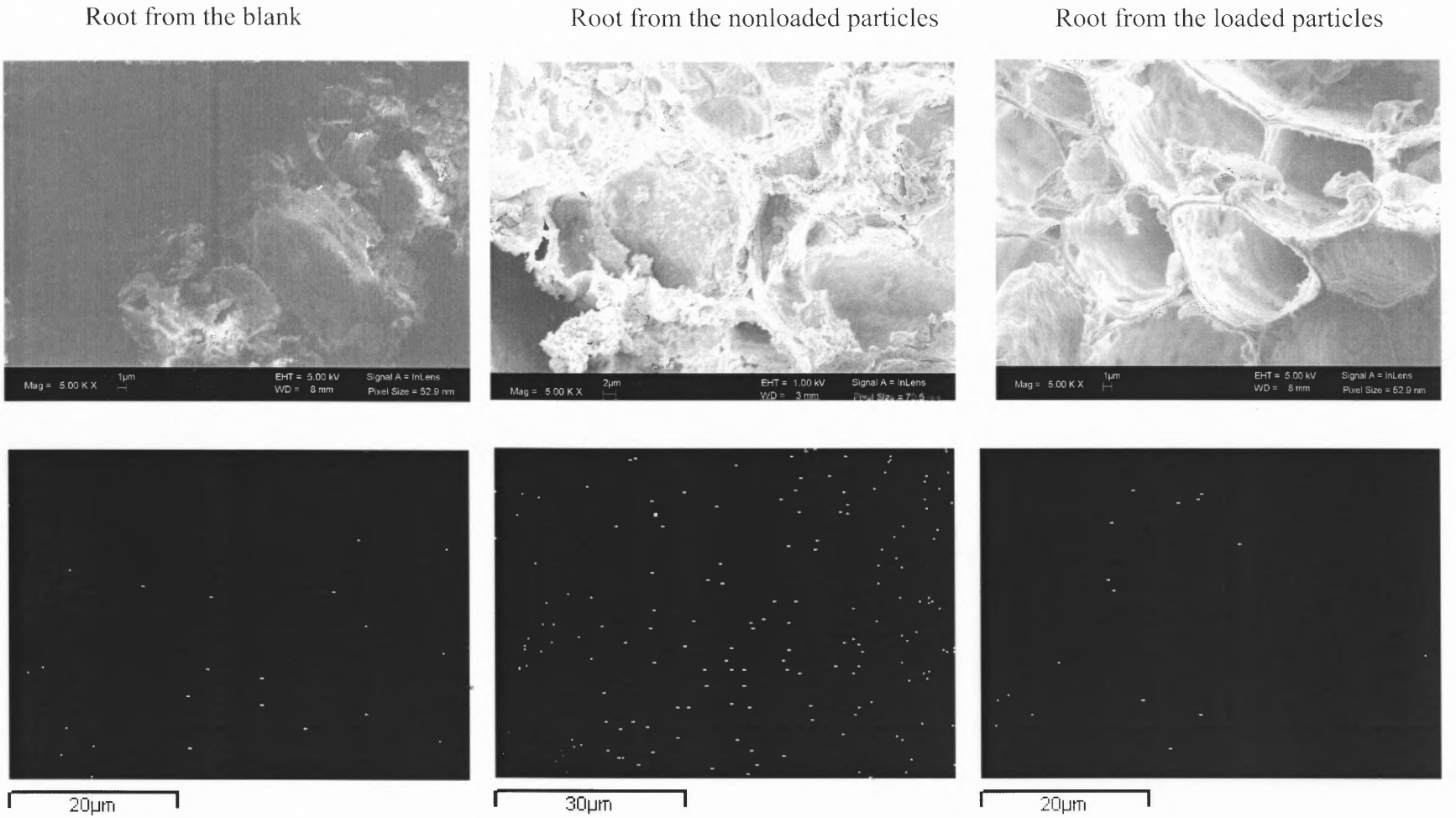


Figure 5.5 The SEM pictures (the upper layer) and the results from the EDS analysis of the element Al (the lower layer). The bright spots in the pictures of the below layer indicate the appearance of the element Al.

Based on the results from this study, it is proposed that 1) the loading of 10.0% ML, 100.0% ML, and 432.4% ML of phenanthrene did not change the size of the alumina nanoparticles; however it may change the surface characteristics of the alumina nanoparticles through interaction with the free hydroxyl groups on the particle surface, 2) the change of the surface characteristics of the alumina nanoparticles changes the phytotoxicity of the particles, possibly through a change in the extent to which the particles may enter the root systems and induce physiological changes of the roots, and 3) the surface characteristics of alumina nanoparticles contribute to the phytotoxicity of the alumina nanoparticles.

Though some preliminary conclusions may be drawn from the results of this study, determination of the detailed mechanisms of how alumina nanoparticles inhibit root growth of the seedlings will require additional study. It will be important to elucidate the contributions of the surface characteristics of the particles to their toxicity. This type of information can provide a scientific basis for pollution control of manmade nanoparticles.

5.2 Phen-Loaded 21-nm Titania Particles

5.2.1 Phytotoxicity Study

The amount of the Phen that was loaded on the particles is 5.13 mg loaded onto 0.5 grams of the titania nanoparticles, resulting in 10.26 mg/g, which is equivalent to 100.0% MML of loading for this particle material. Two categories of loaded particles were studied: the freshly loaded particles, and the aged loaded particles. The aged loaded

particles were freshly loaded particles that had been kept in dark at room temperature for six months.

The toxicity of both freshly Phen-loaded and aged Phen-loaded 21-nm titania particles was investigated by the root elongation test using the *B. oleracea* and the *C. sativus* at 2 mg/ml. The aged Phen and fresh Phen not adsorbed on particles were also tested. The test concentration of Phen was 0.02 mg/ml, which is the same amount as the Phen in the 2 mg/ml dosing of the particle suspensions (refer to Chapter 3, Section 3.2.3 for the procedure for Phen testing). Table 5.6 gives the RE and RRG results.

Compared to the RE of seedlings in the blanks, in all of the titania nanoparticle suspensions tested, only the aged Phen-loaded titania particles were found harmful to seedling root growth, with a mean value of the RE for the *B. oleracea* of 14.6, compared to the 21.7 from the blanks ($p = 0.000$), and for the *C. sativus* of 22.8, compared to the 31.9 from the blanks ($p = 0.000$). There is no significant difference between the RRG for the *B. oleracea* and the *C. sativus* within a concentration.

Statistically significant differences have been found between the RRG of the aged and freshly loaded particles or nonloaded particles ($p = 0.0001$ between the aged and the freshly loaded, and 0.0001 between the aged and the nonloaded), whereas no difference has been found between the RRG of the freshly loaded and the nonloaded particles ($p = 0.443$ for the *B. oleracea*, and 0.540 for the *C. sativus*). The aged Phen didn't show similar effects as the aged Phen-loaded particles ($p = 0.0003$ for the *B. oleracea*, and 0.0001 for the *C. sativus*). This result suggests that either the toxic effect of the aged loaded particles does not come from the aged Phen, or the aged Phen that has been adsorbed on the surface of the particles is different than the aged Phen that has been kept

alone. It was noticed that after six months, the color of the Phen-loaded particles turned to light brown, whereas the aged Phen remained white. Therefore, the latter suggestion may be the reason. The aged Phen that has been kept alone at the same time did not show

Table 5.6 Phytotoxicity of Freshly 100.0% MML Phen-loaded Titania Nanoparticles and 6-month Old Phen-loaded Particles

Toxicity was evaluated by root elongation of *B. oleracea* and *C. sativus* seedlings. Results are expressed as mean ± S.D. and 95% confidence interval. Statistical analysis was performed by comparing the RE values from the blanks and the particle suspensions. Significant difference was reported as the p is smaller than 0.05.

			B. oleracea	*C. sativus*
RE	Nonloaded	Blank	14.0 ± 4.3, 12.3 ~ 15.6	38.4 ± 4.6, 37.0 ~ 39.8
		Particles	16.2 ± 3.9, 14.7 ~ 17.6	37.4 ± 4.4, 35.8 ~ 39.0
			p_1[a] = 0.058, p_2[b] = 0.0585	p_1 = 0.339, p_2 = 0.3389
			R^2 = 0.9316	R^2 = 0.9865
	Freshly loaded	Blank	17.0 ± 5.5, 15.0 ~ 19.0	31.9 ± 5.2, 30.0 ~ 33.7
		Particles	18.6 ± 4.0, 17.2 ~ 20.0	31.7 ± 4.6, 30.0 ~ 33.3
			p_1 = 0.179, p_2 = 0.1788	p_1 = 0.864, p_2 = 0.8643
			R^2 = 0.9685	R^2 = 0.9995
	Aged loaded	Blank	21.7 ± 5.8, 19.6 ~ 23.9	31.9 ± 5.2, 30.0 ~ 33.7
		Particles	14.6 ± 5.7, 12.4 ~ 16.7	22.8 ± 4.6, 21.1 ~ 24.4
			p_1 = 0.000, p_2 = 0.0001	p_1 = 0.000, p_2 = 0.0001
			R^2 = 0.7098	R^2 = 0.5299
	Fresh Phen	Blank	21.7 ± 5.8, 19.6 ~ 23.9	33.0 ± 4.1, 31.6 ~ 34.5
		Phen	21.2 ± 5.7, 19.2 ~ 23.2	31.9 ± 3.1, 30.8 ~ 33.0
			p_1 = 0.726, p_2 = 0.7258	p_1 = 0.228, p_2 = 0.2285
			R^2 = 0.9977	R^2 = 0.9751
	Aged Phen	Blank	21.7 ± 5.8, 19.6 ~ 23.9	33.0 ± 4.1, 31.6 ~ 34.5
		Phen	20.7 ± 5.4, 18.5 ~ 22.9	32.2 ± 3.6, 30.9 ~ 33.5
			p_1 = 0.508, p_2 = 0.5085	p_1 = 0.406, p_2 = 0.4057
			R^2 = 0.9908	R^2 = 0.9880
RRG	Nonloaded		1.15 ± 0.28, 1.05 ~ 1.26	0.97 ± 0.12, 0.93 ~ 1.01
	Freshly loaded		1.10 ± 0.23, 1.02 ~ 1.18	0.99 ± 0.14, 0.94 ~ 1.04
	Aged loaded		0.67 ± 0.26, 0.57 ~ 0.77	0.71 ± 0.14, 0.66 ~ 0.77
	Fresh Phen		0.98 ± 0.26, 0.88 ~ 1.07	0.96 ± 0.09, 0.93 ~ 1.00
	Aged Phen		0.95 ± 0.25, 0.85 ~ 1.05	0.97 ± 0.11, 0.94 ~ 1.01

[a] Calculated from the *Student's t-test*

[b] Calculated from the *one-way ANOVA* procedure

The size of the freshly loaded particles is 264.8 ± 64.6 nm, and the size of the "aged" loaded particles is 364.9 ± 77.1 nm. The sizes of the both loaded particles are about double of the size of the nonloaded particles, which is119.5 ± 58.2 nm

any difference in toxicity compared with the fresh Phen that was kept in dark under vacuum (p = 0.7359 for the *B. oleracea*, and 0.7276 for the *C. sativus*). The FTIR study on the two particle free-forms of Phen does not show any difference in structure between them (data not shown).

5.2.2 FTIR Study

The freshly Phen-loaded particles, the "aged" Phen-loaded particles, as well as the nonloaded 21-nm Titania particles were analyzed by the FTIR. Figure 5.6 gives the spectra that were obtained by subtraction of the spectrum of nonloaded particles from either the spectrum of the freshly loaded particles or the spectrum of the "aged" loaded particles.

A new band near 676 ~ 885 cm^{-1} appeared after the loading of the Phen. The loading of Phen for the freshly loaded particles can decrease the absorbance at 1600 ~ 1700 cm^{-1}, and at 3150 ~ 3700 cm^{-1}, which result in the two inversed bands in the two wavenumber ranges. The two inversed bands however disappeared if the particles were stored six months after they were loaded with Phen, and some broad bands near wavenumbers of 1110 cm^{-1}, 1200 cm^{-1}, 1435 cm^{-1}, and 1637 cm^{-1} appeared. The absorbance at the wavenumber of 1383 cm^{-1} at the same time was enhanced. It is proposed from the FTIR results that new substances were formed on the surface of the particles, and the substances are oxidization products of the particle adsorbed Phen.

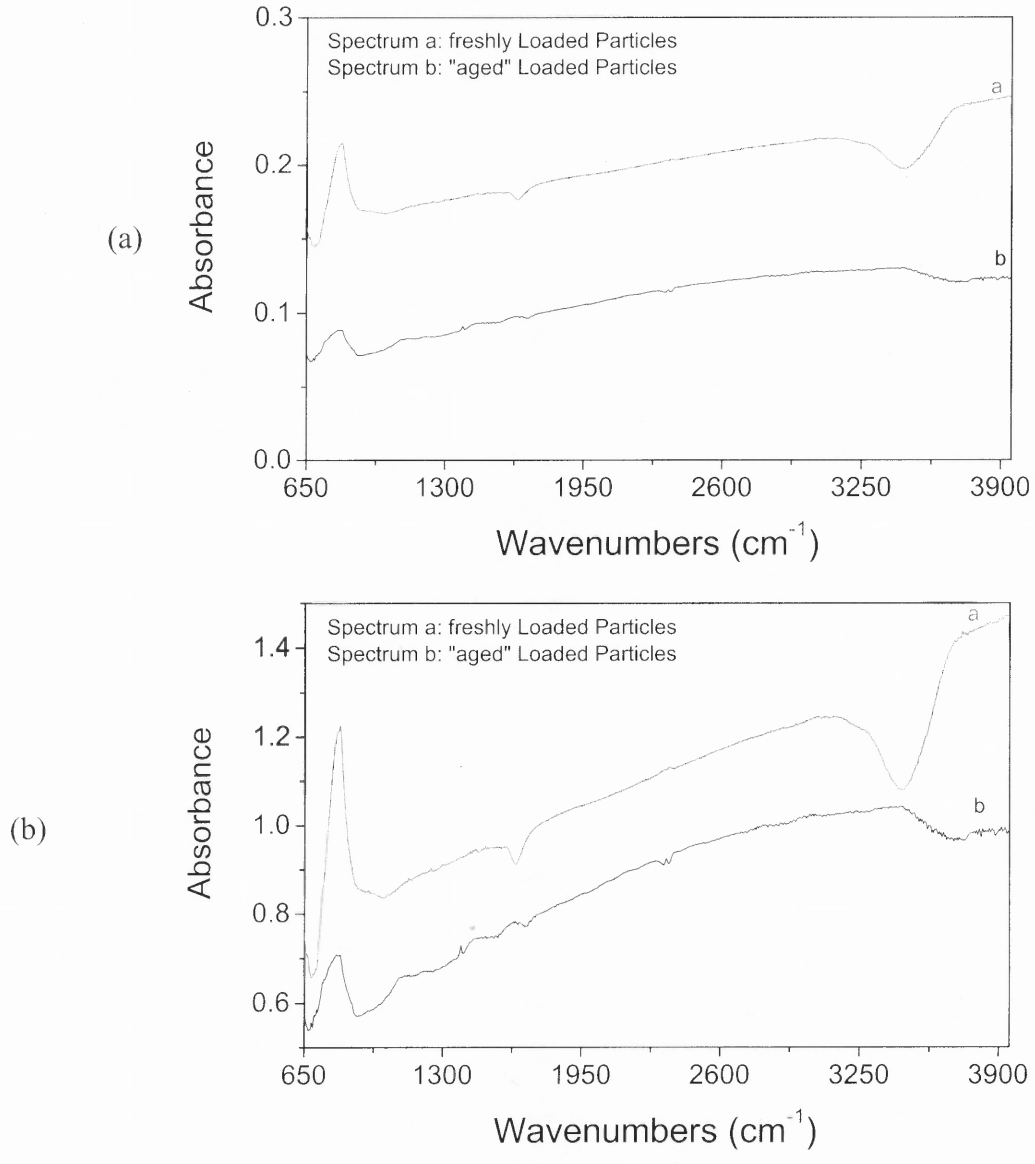


Figure 5.6 FTIR study on the 21-nm Titania particles that were loaded with 100.0% MML of Phen. The FTIR studies were performed on the freshly loaded particles, the loaded particles that have been stored in the dark at room temperature for six months, and the nonloaded particles. The spectra presented here are the results of subtraction of the spectrum of the nonloaded particles from the spectra of loaded particles, (a) the original subtracted spectrum, and (b) the original subtracted spectra eight times enlarged.

5.3 Phen-loaded 14-nm Hydrophilic Silica Particles

5.3.1 Phytotoxicity Study by the Root Elongation Test

The 0.5 grams of particles were loaded with 25.0 mg of Phen to get 50.0 mg/g (equivalent to 90.6% MML) of Phen loading. The toxicity of the loaded particles was investigated by the root elongation test using the *B. oleracea* and the *D. carota* seedlings at 2 mg/ml. The RE and RRG results are given in Table 5.7.

It has been discussed in Chapter 4, Section 4.2, that the 14-nm hydrophilic silica particles can enhance the root growth of the seedlings, and the enhancement effect was seen as an adverse effect. The mechanism that leads to the phytotoxicity for the silica particles was suggested to be different than that of the 13-nm alumina particles because of the unusual nature of the response.

It was found in this study that after being loaded with 50.0 mg/g (i.e., 0.1 mg/ml in the particle suspensions that were being tested, which should be nontoxic since the 0.28 mg/ml of Phen has been tested and found nontoxic), the particles could inhibit the root growth of the seedlings. The average RE of the *B. oleracea* seedlings in the loaded particle suspensions was 18.8 mm, compared to the 22.4 mm of the seedlings in the blank, with $p = 0.009$. The average RE of the *D. carota* seedlings in the loaded particle suspensions was 11.8 mm, compared to the 13.7 mm of the seedlings in the blank, with $p = 0.0002$. The RRG from the loaded particles is significantly different than the RRG from the nonloaded particles, which is reasonable since the RRG from the nonloaded particles is larger than the blanks.

Table 5.7 The RE and RRG of Seedlings Exposed to 2 mg/ml of the 14-nm Hydrophilic Silica Particles Loaded with the 90.6% MML of Phen

The exposure was done in dark at 25 ± 1 °C for 24 hrs. The results reported are the mean value ± S.D. Statistically significant difference is reported as the p smaller than 0.05.

		B. oleracea	*D. carota*
RE	Blank	22.4 ± 6.2, 20.1 ~ 24.6	13.7 ± 1.9, 13.0 ~ 14.4
	Loaded particles	18.8 ± 3.7, 17.4 ~ 20.1 p_1[a] = 0.009, p_2[b] = 0.00903 R^2 = 0.8864	11.8 ± 1.6, 11.2 ~ 12.4 p_1 = 0.0002, p_2 = 0.6755 R^2 = 0.6755
RRG	Nonloaded particles	1.12 ± 0.37, 0.99 ~ 1.25	1.00 ± 0.24, 0.91 ~ 1.08
	Loaded particles	0.84 ± 0.17, 0.78 ~ 0.90 p_1[c] = 0.0004, p_2[d] = 0.000401 R^2 = 0.8042	0.86 ± 0.12, 0.82 ~ 0.91 p_1 = 0.0092, p_2 = 0.009155 R^2 = 0.8848

[a] Calculated from the *Student's t-test*; [b] Calculated from the *one-way ANOVA* procedure
[c] Based on comparison between nonloaded and loaded, calculated from the *Student's t-test*
[d] Based on comparison between nonloaded and loaded, calculated from the *ANOVA* procedure
The size of the loaded particles is 207.1 ± 47.1 nm, which is not dramatically different than the size of 215.7 ± 56.3 nm of the nonloaded particles

This finding is different than that had been discovered in the studies on the Phen-loaded 13-nm alumina particles, in which the loaded particles were found to be nontoxic whereas the nonloaded particles were determined to be phytotoxic. These disagreeing observations may be explained as the result of different mechanisms of action by these two different particles.

5.3.2 FTIR Study

The silica particles that had been loaded with 90.6% MML Phen and the nonloaded particles were studied by FTIR. The spectrum of the nonloaded particles was subtracted from the spectrum of the loaded particles (Figure 5.7).

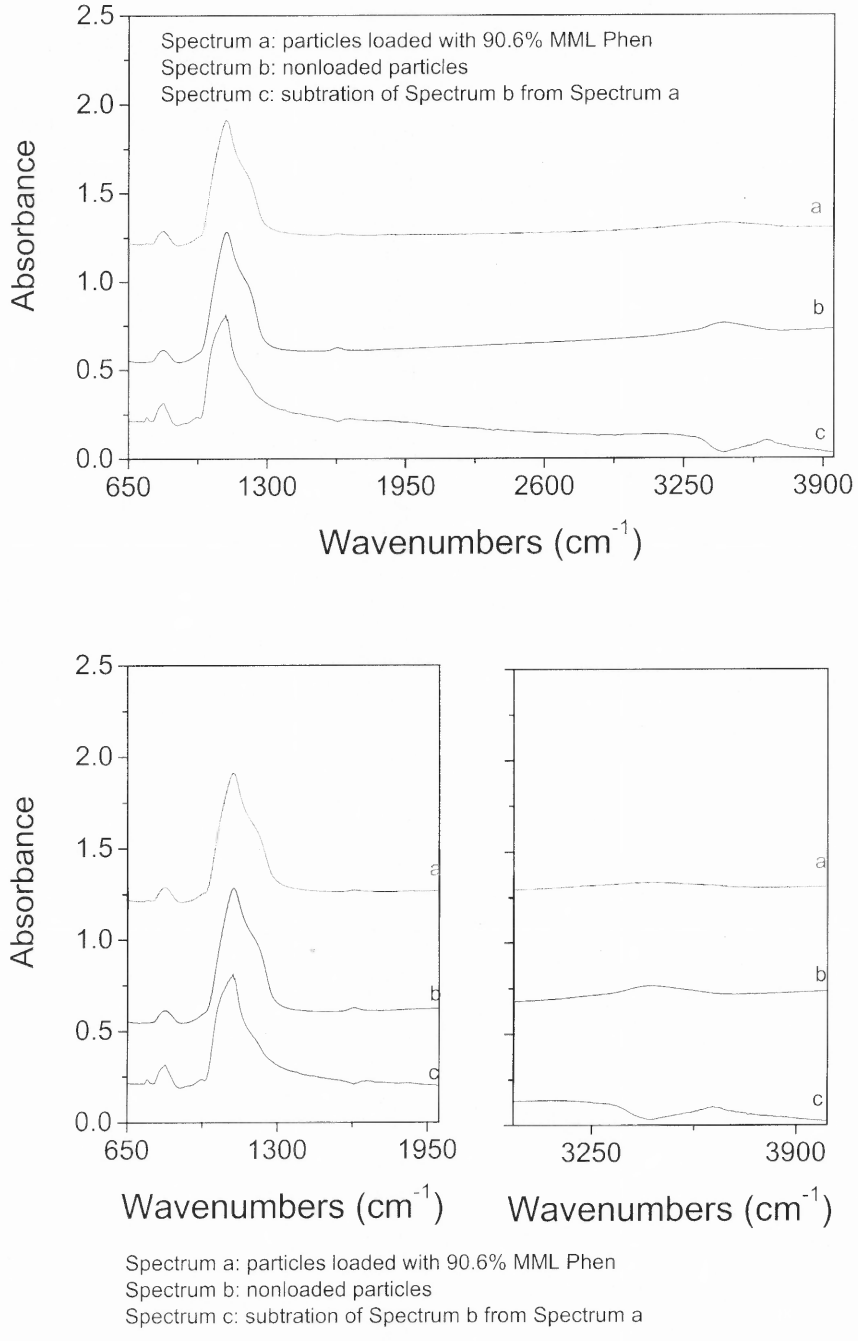


Figure 5.7 FTIR spectra of 14-nm hydrophilic silica particles.

It was noticed that after the particles were loaded with Phen, the absorbance near 800 cm^{-1} and 1100 cm^{-1} was enhanced, whereas the absorbance in the range of 3300 ~ 3500 cm^{-1} was reduced (see Spectrum c in Figure 5.7). Two bands near 730 cm^{-1} and 975 cm^{-1} appeared, which were assigned to the absorption of the surface adsorbed Phen.

5.4 Phen-Loaded 161.2-nm Hydrophilic Spherical Silica Particles

5.4.1 Phytotoxicity Study by the Root Elongation Test

The 1.0 grams of 161.2-nm silica particles were loaded with 20.2 mg Phen to get 40.4 mg/g (i.e., 99.6% MML) of Phen loading. This amount of Phen was selected because the 100% MML theoretically gives a theoretically complete cover of Phen layer on the particle surface, which can become a good study object for both the investigation of the interaction between the adsorbed Phen and the particles and the toxicity study of the loaded particles.

The phytotoxicity of the loaded and nonloaded particles in this study of Phen-loaded 161.2-nm hydrophilic spherical silica particles was investigated by the *B. oleracea* seedling root elongation test. The test concentration of the particle suspensions was 20 mg/ml, because at 20 mg/ml the toxic effect of the particles was prominent under the experimental conditions. The "nonloaded" particles for this study were treated with 4 ml of acetone, just as what had been done in the investigation of the influence of the Phen-loading on the particles previously studied in this way. Table 5.8 gives the RE and RRG results of the phytotoxicity test.

Table 5.8 Result of Acetone Treatment or Phen-loading on the Phytotoxicity of the 161.2-nm Hydrophilic Spherical Particles

The results are reported as the mean value ± S.D. Statistical difference is reported as the p is smaller than 0.05.

RE (mm)	Blank	18.9 ± 5.7, 16.9 ~ 21.0
	Nonloaded but acetone treated	18.0 ± 5.0, 16.2 ~ 19.8 p_1[a] = 0.517, p_2[b] = 0.5171, R^2 = 0.9925
	99.6% MML loaded	15.3 ± 4.3, 13.8 ~ 16.9 p_1 = 0.00084, p_2 = 0.00837, R^2 = 0.8720
	99.6% MML loaded vs. Acetone treated	p_1 = 0.0336, p_2 = 0.03360, R^2 = 0.9205
RRG	Original particles	0.62 ± 0.19, 0.54 ~ 0.69
	Nonloaded but acetone treated	0.95 ± 0.26, 0.85 ~ 1.05
	99.6% MML loaded	0.81 ± 0.22, 0.73 ~ 0.89
	Original vs. Nonloaded but acetone treated	p_1 = 0.000, p_2 = 0.0001, R^2 = 0.6518
	Original vs.99.6% MML loaded	p_1 = 0.0016, p_2 = 0.00155, R^2 = 0.8233

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure.

The 161.2-nm hydrophilic spherical particles turned less toxic or even nontoxic after being treated with acetone (mean RE = 18.0 mm, compared to the 18.9 mm from the blanks, and the p = 0.5171), unlike the other particles that have been discussed in the above sections. The toxicity of the particles increased compared to the particles treated with acetone if it is loaded with 99.6% MML of Phen (mean RE = 15.3 mm, compared to the 18.0 mm from the acetone-treated particles, with the p = 0.0336), but decreased compared to the original particles for which the toxicity has been tested previously (see Chapter 4, Section 4.4) with the p = 0.00155. A significant difference was also found between the original particles and the acetone-treated particles (p = 0.0001).

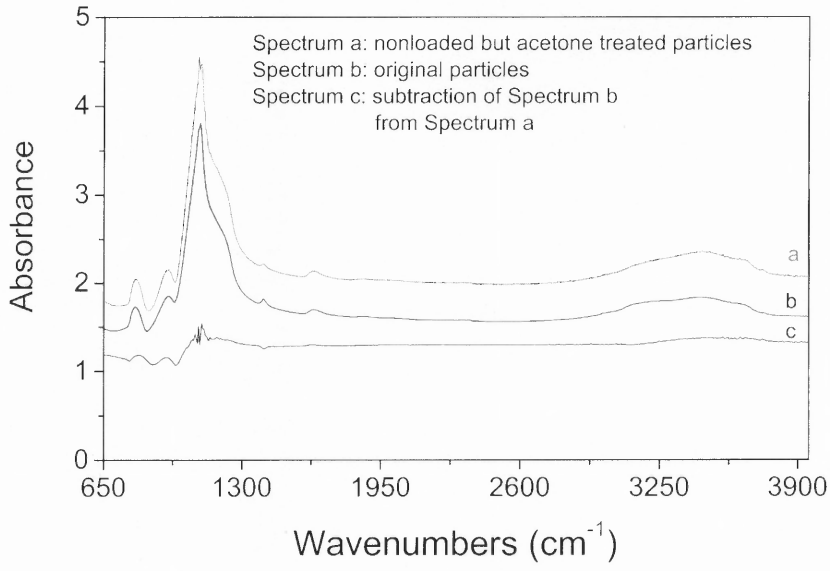
The results are very hard to explain based on the toxicity tests alone. The size of the loaded particles was determined by the Coulter N4+, which is 322.5 ± 29.0 nm after being treated with acetone, and 325.2 ± 23.7 nm after being loaded with Phen (compared with 161.2 nm of the original particles). FTIR studies at the same time were performed to investigate whether the surface characteristics of the particles had been changed by the acetone treatment, which is discussed in the following Section.

5.4.2 FTIR Study

The particles were studied by FTIR following the procedure that was given in Section 3.2.1. The FTIR spectra of the nonloaded but acetone treated particles, as well as of the original particles were compared [Figure 5.8 (a)]. The FTIR spectrum of the Phen-loaded particles was given in Figure 5.8 (b), along with the spectrum of the original particles and the spectrum obtained by subtraction.

The results from the FTIR study showed that after being treated with acetone, the absorbance around 805 cm^{-1} and 945 cm^{-1} was enhanced. This change however doesn't happen after the Phen–loading. Both the acetone treatment and the Phen – loading reduce the absorbance near 1400 cm^{-1} where a reversed peak appears at this wavenumber in either case of subtraction.

(a)



(b)

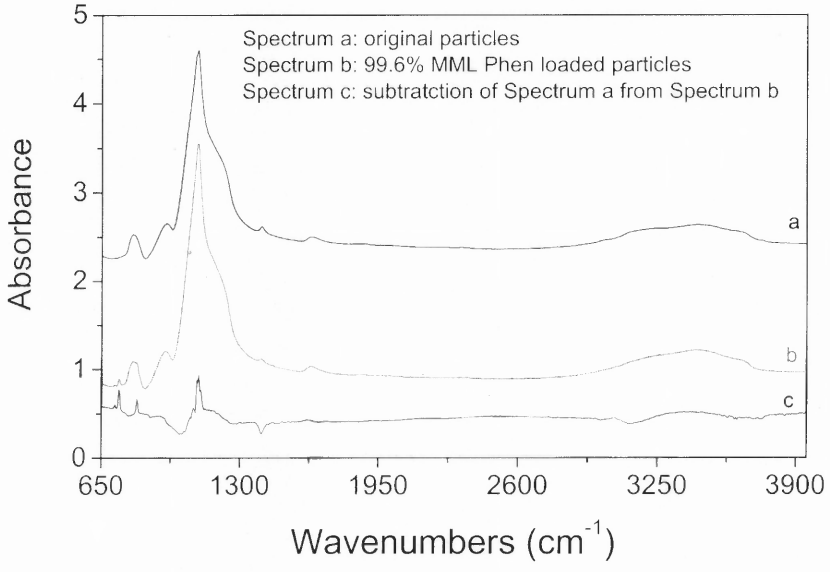


Figure 5.8 FTIR study of the 161.2-nm hydrophilic spherical silica particles: (a) comparison between the spectrum of the original particles and the spectrum of the acetone – treated particles; and (b) comparison between the spectrum of the original particles and the 99.6% MML Phen loaded particles.

5.5 Phen-Loaded Particles with Sub-micron and Micron Sizes

The effect on plant root growth of the sub-micron and micron particles after being loaded with ~ 100% MML of Phen was studied using the *B. oleracea* seedlings. Compared to their nanometer scaled siblings, a much smaller amount of Phen was required for the ~100% MML loading: for the 667.6-nm hydrophilic spherical silica particles, it is 1.50 mg/g (94.9% MML, 1.50 mg phenanthrene to 1.0 grams particles in this study), for the 0.96-μm titania particles, the amount is 3.30 mg/g (97.9% MML, 3.30 mg phenanthrene to 1.0 grams particles in this study), and for the 1.0-μm alumina particles, 0.52 mg/g (92.6% MML, 0.52 mg Phen to 1.0 grams of particles in this study) was loaded. Similar quantities of the particles were also treated with 4 ml acetone to get the nonloaded particles for the study. The root elongation test was performed at the concentrations of 20 mg/ml, which was the largest concentration of the submicron and micron particles that has been toxicity-tested. The RE and RRG results are given in Table 5.9.

The toxic effect of the nanoparticles was changed more or less by the Phen loading, as discussed in the above sections, except for the freshly loaded 21-nm titania that showed no difference in toxicity. The Phen loading in the case of the particles with sub-micron or micron sizes however does not change the root growth effect of the particles, and the loaded particles remained nontoxic to the root growth of the seedlings. The acetone treatment at the same time did not change the effect of the particle on the root growth either. This is similar to what has been found with most of the nanoparticles except the 161.2-nm silica particles.

5.6 Summary and Conclusions

The phytotoxicity of the Phen-loaded particles was studied in this chapter. Results demonstrate that the loading of the Phen can change the toxic effect of the nanoparticles, but not the effect of the particles in the sub-micron or micro size scales.

The inhibitory effect of the 13-nm alumina, if loaded with 10.0% or 100.0% MML of Phen particles, was decreased significantly. This phenomenon is postulated to come from changes of the particle surface characteristics in which the free hydroxyl groups on the particle surfaces may play an important role.

The Phen loading of the 14-nm hydrophilic silica changed the enhancement effect of the particles into an inhibition effect. This toxicity change does not result from the change of particle size, because after being loaded with Phen, the size of the particles remained almost the same. FTIR study revealed that after being loaded with Phen there were some changes on the particle surface. No evidence available to date however can support whether or not it caused the toxicity change of the particles. The mechanisms that cause the adverse effect were postulated to be different than those of the 13-nm alumina particles. These two particles may interact with the plant seedlings and damage the root growth in different ways. The reasons for these differences need more exploration.

The freshly Phen-loaded 21-nm titania particles were found to be just as nontoxic as the nonloaded 21-nm titania. Once the loaded particles were stored for some time (6 months in this study), they showed toxicity. The adsorbed Phen may change to some toxic form, which leads to the toxicity of the aged Phen-loaded particles, even though the storage was in the dark. This phenomenon may result from the fact that the titania nanoparticles are an oxidative catalyst.

Table 5.9 Phytotoxicity Study Results of the Sub-micron and Micron Sized Particles Loaded with ~ 100% MML of Phen

The results are reported as the mean value of the RE/RRG ± S.D, with the 95% confidence interval. Statistical analysis was performed between the blank controls and the samples, and unless otherwise stated is between the samples. The concentration tested was 20 mg/ml, and the plant species used is the *B. oleracea*.

		RE	RRG
	Blank	22.4 ± 6.2, 20.1 ~ 24.6	1.00 ± 0.28, 0.90 ~ 1.10
	Acetone treated	23.3 ± 5.8, 21.0 ~ 25.6 p_1[a] = 0.584, p_2[b] = 0.5835, R^2 = 0.9942	1.04 ± 0.26, 0.94 ~ 1.14
	94.9% MML loaded	21.5 ± 5.5, 19.5 ~ 23.5 p_1 = 0.566, p_2 = 0.5657, R^2 = 0.9941	0.96 ± 0.24, 0.87 ~ 1.05
667.6-nm silica	Original particles	-	0.98 ± 0.35, 0.85 ~ 1.83
	Acetone treated vs. 94.9% MML loaded	p_1 = 0.246, p_2 = 0.2465, R^2 = 0.9743	-
	Acetone treated vs. original particles	-	p_1 = 0.461, p_2 = 0.4612 R^2 = 0.9897
	Acetone treated	21.9 ± 6.2, 19.6 ~ 24.3 p_1 = 0.246, p_2 = 0.2465, R^2 = 0.9743	0.98 ± 0.28, 0.88 ~ 1.08
0.96-μm titania	97.9% MML loaded	23.5 ± 9.8, 20.0 ~ 27.0 p_1 = 0.593, p_2 = 0.5928, R^2 = 0.9950	1.05 ± 0.44, 0.89 ~ 1.21
	Original particles	-	0.90 ± 0.20, 0.82 ~ 0.97
	Acetone treated vs. 97.9% MML loaded	p_1 = 0.475, p_2 = 0.4754, R^2 = 0.9907	-
	Acetone treated vs. original particles	-	p_1 = 0.210, p_2 = 0.2095 R^2 = 0.9715
	Acetone treated	21.3 ± 8.0, 18.3 ~ 24.3 p_1 = 0.583, p_2 = 0.5826, R^2 = 0.9943	0.95 ± 0.36, 0.82 ~ 1.09
1.0-μm alumina	92.6% MML loaded	22.0 ± 6.2, 19.8 ~ 24.3 p_1 = 0.835, p_2 = 0.8354, R^2 = 0.9992	0.98 ± 0.28, 0.88 ~ 1.08
	Original particles	-	0.96 ± 0.26, 0.86 ~ 1.05
	Acetone treated vs. 92.6% MML loaded	p_1 = 0.707, p_2 = 0.7069, R^2 = 0.9974	-
	Acetone treated vs. original particles	-	p_1 = 0.956, p_2 = 0.9561 R^2 = 0.9999

[a] Calculated from the *student's t-test*
[b] Calculated from the *one-way ANOVA* procedure

Both the acetone treatment and the Phen-loading decreased the toxic effect of the 161.2-nm silica particles significantly. Statistical differences were also found between the phytotoxic effects of the acetone treated particles and particles loaded with Phen, and the latter was found more toxic than the former. It is postulated that the Phen-loading on the particles results in more bioavailability of the Phen. The non-toxicity of Phen that has been found in this study may come from the non-bioavailability of the Phen, which has also been suggested in the literature (Hubner et al., 2000; Becker et al., 2002). FTIR study results alone did not provide any evidence that can help to explain the toxicity change of the 161.2-nm hydrophilic spherical silica particles after they were loaded with Phen. The size of the particles almost doubled in this case after being treated with acetone or loaded with Phen. The 161.2 nm silica particles were made locally as part of an experimental program. The particles were made to be mono-dispersed. The acetone treatment and Phen-loading changed the status of mono-dispersion of the particles, which may result in the reduction of the particle toxicity.

The results from this chapter suggest that the study of the toxicity change of the particles after being loaded with chemicals is applicable to investigation of the mechanisms of particle-induced injury to the living organisms. The study of the mechanisms in most of the published literature focuses on the living organisms, not the particles. In fact, good understanding of the particle properties can help to discover the underlying mechanisms that lead to particle-induced injury.

CHAPTER 6

INVESTIGATION OF PARTICLE PROPERTIES THAT CONTRIBUTE TO PARTICLE INDUCED TOXIC EFFECTS

The phytotoxicity of manufactured particulate materials was investigated in Chapter 4. The 13-nm alumina particles, the 14-nm hydrophilic silica particles, and the 161.2-nm hydrophilic spherical silica particles were found to have adverse effects on the plant seedling growth. The influence of phenanthrene loading on the toxicity of the particles was studied in Chapter 5 to investigate the role taken by particle surface associated chemical species in the particle phytotoxicity. Phen-loading can decrease the phytotoxicity of the 13-nm alumina particles, as well as that of the 161.2-nm silica particles. The root growth enhancement effect of 14-nm hydrophilic silica particles was changed into inhibitory effect after the Phen-loading. The particle properties that may contribute to the particle-induced toxic effects are examined in this chapter.

6.1 Particle Size

Particles with different sizes but within the same material category, i.e., same chemical composition (i.e., TiO_2, SiO_2 and Al_2O_3), were obtained in this study. The experimental design was to investigate the contribution of the particle size to the particle-induced adverse effect. The phytotoxicity of the particles with different sizes but within the same chemical category is given as the RRG values in Table 6.1. The effects on the plant seedling root growth were compared at 20 mg/ml for the particles for which the phytotoxic effects have been investigated. The *Student's t-test* and the *one-way ANOVA*

procedure were performed to compare the RRG results from the testing of the particles with different sizes. The significant difference was reported as the possibility of the null hypothesis (p) being smaller than 0.05 as usual.

Table 6.1 The RRG Values of Plant Seedlings Exposed to 20 mg/ml of the Particle Suspensions – the Investigation of the Contribution of the Particle Size to the Phytotoxic Effect of the Particles

The RRG values were reported as the mean value of the RRG results from the root elongation tests using different plant species ± S.D.

		RRG
Alumina	13-nm, aggregate size: 201.0 ± 74.69 nm	0.56 ± 0.03
	1.00 ± 0.056 μm	0.97 ± 0.014
	13-nm vs. 1.00 μm	p_1[a] = 0.000, p_2[b] = 0.0001, R^2 = 0.4428
Silica	14-nm, aggregate size: 215.7 ± 56.3 nm	1.18 ± 0.04 (enhancement)
	161.2 ± 45.6 nm	0.69 ± 0.93
	667.6 ± 7.89 nm	1.00 ± 0.03
	14-nm vs. 161.2-nm	p_1 = 0.000, p_2 = 0.0001, R^2 = 0.5141
	14-nm vs. 667.6-nm	p_1 = 0.000, p_2 = 0.0005, R^2 = 0.9145
	161.2-nm vs. 667.6-nm	p_1 = 0.000, p_2 = 0.0001, R^2 = 0.6369
Titania	21-nm, aggregate size: 119.5 ± 58.2 nm	0.93 ± 0.02
	0.96 ± 0.009 μm	0.94 ± 0.05
	21-nm vs.0.96 μm	p_1 = 0.903, p_2 = 0.9026, R^2 = 1.000

[a] Calculated from the *Student's t-test*
[b] Calculated from the *one-way ANOVA* procedure
Refer Table 3.2 on page 40 for the explanation of particle sizes

It was demonstrated for the alumina and silica particles, that smaller particles (within the same material) have larger toxicity. It cannot be concluded though that the size of the particles alone decides the toxic effect of the particles. The example is the 21-nm titania, for which the mean aggregate size is 119.5 nm, which is similar to the size of the 161.2-nm silica particles. The 21-nm titania however was not found to be toxic to the root growth, and its effect on the root growth was determined to be very similar to the effect of the titania particles with a mean size of 0.96 μm.

6.2 Specific Surface Area of the Particles

This study included particles with different specific surface areas and the same chemical composition, as well as particles with similar specific surface areas and different chemical composition. Table 6.2 gives the mean RRG values of the particles according to their specific surface area.

Table 6.2 The Mean RRG Values and the Specific Surface Areas of the Particles

The RRG values were from the root elongation tests at 20 mg/ml of the particle suspensions.

		Specific surface area m^2/g	RRG
Alumina	13-nm	103.9681	0.56 ± 0.03
	1.00 μm	1.9449	0.97 ± 0.014
Silica	14-nm	200	1.18 ± 0.04 (enhancement)
	161.2-nm	146.9722	0.69 ± 0.93
	667.6-nm	5.7376	1.00 ± 0.03
Titania	21-nm	38.0907	0.93 ± 0.02
	0.96-μm	12.2273	0.94 ± 0.05

The phytotoxicity is similar for the particles with specific surface area larger than 100 m^2/g, including the 13-nm alumina particles, the 14-nm silica particles, and the 161.2-nm silica particles, although significant differences in the RRG values can be determined among the particle types. No toxic effects have been found for other particles with specific surface much smaller than 100 m^2/g, including the 21-nm titania, 667.6-nm silica, 0.96-μm titania, and 1-μm alumina. The data demonstrates that among the materials studied, larger specific surface area is related to increased phytotoxicity. The specific surface area is more important than the particle size where the toxic effect of the particles is concerned.

6.3 The Mass Concentration and Number Concentration of the Particles

The influence of the mass concentration (mg/ml) on the toxicity of the particles was studied in Chapter 4. It was stated that the toxic effect of the particles is dose-dependent.

The number concentration (numbers of particles per ml) of the particles may also play an important role in the toxicity of the particles. For example, the number concentration of the 13-nm alumina particles is about 4.52 fold larger compared to that of the 21-nm titania particles (which was calculated from the density and the diameter of the particles). The RRG data showed that the toxicity of the 13-nm alumina particles is about 1.8 fold greater compared to that of the 21-nm titania, which may suggest that the toxicity of the particles increases with the increase of the number concentration of the particles. No conclusion can be drawn from the available evidence though because the specific

surface area values of these two particles are different. The difference in their toxicity may result from the difference of the specific surface area, or some function related to that.

6.4 Particle Surface-Associated Chemical Species

Phenanthrene was loaded on the particles in Chapter 5. The phytotoxic effect of the particles was studied after the Phen-loading. The results showed that the Phen-loading could decrease the phytotoxicity of some of the particles that have been found toxic to root growth, including the 13-nm alumina particles and the 161.2-nm hydrophilic spherical silica particles. The root growth enhancement effect of 14-nm hydrophilic silica particles was changed into root inhibitory effect after Phen-loading. The freshly Phen-loading failed to change the observed lack of phytotoxicity of the 21-nm titania particles though. It was postulated however, from the results of a long time storage study that the titania particles were able to accelerate the reaction (probably oxidation) of the particle surface adsorbed–Phen, which resulted in the formation of a more toxic chemical species.

The adsorption of chemical species on the surface of the particles is suggested to change the particle surface characteristics according the FTIR study results (for example, the probable modification of the free hydroxyl group in the case of the 13-nm alumina particles), and the bioavailability of the chemical species, which results in changes of the particle toxicities.

6.5 Summary and Conclusions

The contribution of particle properties including particle size, particle specific surface area, mass concentration (mg/ml), number concentration (in milliliter of water), and surface-associated chemical species were evaluated in this chapter. It was suggested that where the phytotoxicity of the particles is concerned, the particle specific surface area, the mass concentration, and the particle surface – associated chemicals are important. It was determined that particle size, although important, was not the deciding factor. The particle surface–associated chemicals are important partly because they can change the particle surface characteristics, which were found to be crucial to the particle toxicity.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The toxicity as well as the underlying responsible characteristics of manufactured particulate materials on plant seedling root growth was studied in this dissertation. The manufactured particles within the nanometer scale range were used as models to investigate the properties that are important to the toxicity of airborne nanoparticles.

The manufactured particles studied in this dissertation include the AluC from Degussa (13-nm alumina), the Cab-O-Sil M5 from Cabot (14-nm hydrophilic silica), the Aeroxide TiO_2 P25 from Degussa (21-nm titania), 0.96-μm titania and 1-μm alumina from Atlantic Equipment Engineers, 667.6-nm hydrophilic spherical particles, and a locally made hydrophilic spherical silica particles with the diameter of 161.2 nm.

The phytotoxicity of the particles was investigated by the root elongation test, which followed the EPA recommended standard procedure with slight modifications. The particles were suspended in Milli-Q water at a series of concentrations. The plant seeds, including seeds of *B. oleracea*, *C. sativus*, *A. sativa*, *G. max*, *Z. mays*, and *D. carota*, were cultured into seedlings at 25 ± 1 °C in the dark before exposure to the particle suspensions. The exposure to the particle suspensions was done at 25 ± 1 °C in the dark for 24 hrs (for long time exposure, 48 hrs and 72 hrs were also included). Milli-Q water was used as the control blank.

Adverse effects on root growth were found for the 13-nm alumina particles, the 14-nm hydrophilic silica particles, and the161.2-nm hydrophilic spherical particles,

among which the 13-nm alumina particles and the 161.2 hydrophilic spherical particles inhibited the root growth, whereas the 14-nm hydrophilic silica enhanced the root growth. The phytotoxicity was determined to be dose-dependent, with the IC_{10} (concentration that causes 10% of inhibitory effects) of 0.281 mg/ml, 1.803 mg/ml, and 5.420 mg/ml for the 13-nm alumina particles, the 161.2-nm hydrophilic spherical silica particles, and the 14-nm hydrophilic spherical silica particles, respectively. Long time exposure (24 hrs, 48 hrs and 72 hrs for the 13-nm alumina, and 24 hrs and 48 hrs for the 14-nm silica and 161.2-nm silica) showed that the adverse effect of the particles happens in the first 24 hrs of exposure under these experimental conditions.

The 21-nm titania particles, the 667.6-nm hydrophilic spherical silica particles, the 0.96-μm titania particles, and the 1.00-μm alumina particles were not found to have any effect on the plant seedling growth compared to the blank control. Note that particles with similar sizes and chemical compositions have been shown to cause abnormal physiological change in both human cell *in vitro* tests and animal *in vivo* tests (Churg et al., 1998; Renwick et al., 2001; Stearns et al., 2001). This difference suggests that the plant root cells respond differently to the particle toxicity than do animal cells.

The influence of chemical species on the particle surface on seedling growth was investigated using phenanthrene (Phen) as the study chemical. Phen was dissolved in acetone, and loaded onto the particles. Both the Phen–loaded particles and the nonloaded particles were analyzed by FTIR, which is a powerful technique to determine particle chemical compositions as well as particle surface characteristics. The phytotoxicity of the loaded particles as well as of the nonloaded particles were determined by the root elongation test following the same procedure that has been used for the investigation of

the phytotoxicity of the particles. The results from the phytotoxicity tests demonstrate that Phen-loading can change the toxicity of the particles.

The loading of 10.0% and 100.0% MML of Phen of the 13-nm alumina particles decreased the phytotoxicity of the particles significantly, to the degree that the particles actually showed similar effects on seedling growth as did the blank control. The FTIR study on the Phen-loaded particles revealed that the decrease of the phytotoxicity of the 13-nm alumina particles might be associated with the loss of the free hydroxyl groups on the particle surfaces. The results from the phytotoxicity determination study on the DMSO-treated particles also supported this. It was observed by SEM/EDS that fewer particles were observed within the plant roots if the particles had been loaded with the 10.0% MML of Phen, than were seen with the nonloaded particles.

The 100.0% MML Phen loading on the 14-nm hydrophilic silica particles changed the root elongation enhancement effect of the particles into an inhibition effect. The mechanisms that cause the toxicity of the particles were postulated to be different for the 13-nm alumina particles and the 14-nm hydrophilic spherical silica particles.

The Phen loading on the 21-nm titania particles did not change the nontoxic properties of the particles if the Phen were loaded just before the tests for phytotoxicity. The particles however, if after being loaded with 100.0% MML of Phen and were kept for six months in the dark, demonstrated high toxicity to the seedling growth. This phenomenon was postulated to come from an oxidation reaction of the Phen that had been adsorbed on the 21-nm titania particles since the titania particles are a known oxidation reaction catalyst.

The phytotoxic effect of the 161.2-nm hydrophilic spherical silica particles disappeared after the particles were treated with acetone, which were the nonloaded particles in the above discussion. The loading of Phen however increased the toxic effect of the particles compared to the nonloaded particles, although it decreased the toxicity of the particles if compared to the original particles. It was postulated that the acetone treatment finally changed the surface characteristics of the particles.

The 100.0% MML Phen loading did not change the nontoxic characteristics of the particles with sub-micron and micron sizes.

The toxicity data, along with the physical and chemical properties of the manufactured particulate materials were compared to determine which particle properties are important to the toxic effects of the particles. The results indicate that:

1) Larger mass concentration (mg/ml of Milli-Q water) of particles that are toxic results in more toxicity,

2) Size of the particles alone cannot explain why the particles are toxic, and are not the sole factor that accounts for particle toxicity. The reason: particles with similar sizes, including the 13-nm alumina particles, the 14-nm silica particles, and the 21-nm titania particles do no have similar phytotoxicity. On the contrary, the former two have similar phytotoxicity as does the 161.2-nm silica particles,

3) The specific surface area of the particles may play an important role in the toxic effects caused by the particles. The particles with specific surface areas larger than 100 m^2/g were toxic to the root growth of the seedlings, whereas the particles with specific surface area much smaller than 100 m^2/g were not found to be toxic to the root growth of the seedlings,

4) The number concentration (numbers of particles in milliliter of Milli-Q water) of the particles is possibly a particle property that contributes to the toxicity of the particles. At the same mass concentration (e.g., 20 mg/ml), the number concentration of 13-nm alumina particles is higher than that of the 21-nm titania particles (about 4.52 fold), and the 13-nm alumina particles were phytotoxic according to this study, whereas the 21-nm titania particles were not toxic,

5) Chemical species on particle surfaces are found to be important to the particle induced–toxic effects. The adsorption of the chemicals on the particle surface are shown to change the toxicity of the particles significantly,

6) FTIR study results showed that particle surface characteristics might be changed after the loading of phenanthrene, which suggests that particle surface characteristics must be considered if the particle toxicity is concerned, and

7) The change of particle surface characteristics may result in change of particle toxicity, which can offer help to the control of particle pollution.

The results from this study demonstrate that the particle size alone does not account for the toxic effect of the particles. This helps to provide the scientific information to indicate the application of nanoparticles, as well as the development of the nanotechnology may be completely acceptable to the environment.

The toxicity of some nanoparticles (e.g., the 13-nm alumina particles) was reduced significantly by changing the particle surface characteristics, which indicates that the particle surface characteristics of particles may play an important role in the particle toxicity. This suggests one method for possible control of the particle pollution.

The data collected in this study provide information that is important to the establishment of particle pollution standards and regulations.

APPENDIX A
PROCEDURE FOR ONE-WAY ANOVA ANALYSIS – AN EXPAMPLE

This example is the phytotoxicity test on the 24-hr exposure to 20 mg/ml and 2 mg/ml 21-nm titania particles using *C. sativus* seedlings.

Raw data (the numbers in **black** is the average values):

20 mg/ml

Group 1

Before Exposure mm	After Exposure mm	RE mm	RRG*
10.9	44.9	34.0	0.884495
18.5	58.3	39.8	1.03538
15.2	46.1	30.9	0.80385
19.5	64.5	45.0	1.170656
7.1	35.0	27.9	0.725806
8.5	39.1	30.6	0.796046
16.2	59.5	43.3	1.126431
20.9	66.5	45.6	1.186264
9.5	33.0	23.5	0.611342
18.5	58.5	40.0	1.040583
		36.06	**0.938085**

* The RE for the blank was 38.4 mm. 38.44 mm was used for more accurate calculation of RRG.

Group 2

Before Exposure mm	After Exposure mm	RE mm	RRG
13.0	49.6	36.6	0.952133
12.2	52.0	39.8	1.03538
8.0	42.5	34.5	0.897503
9.0	30.5	21.5	0.559313
8.1	45.1	37.0	0.962539
9.0	37.6	28.6	0.744017
11.5	46.9	35.4	0.920916
17.5	58.5	41.0	1.066597
9.8	49.9	40.1	1.043184
9.1	45.5	36.4	0.94693
		35.09	**0.912851**

2 mg/ml

Group 3

Before Exposure mm	After Exposure mm	RE mm	RRG
9.5	45.4	35.9	0.933923
7.0	25.3	18.3	0.476067
10.2	51.9	41.7	1.084807
9.0	48.2	39.2	1.019771
8.0	27.0	19.0	0.494277
10.0	49.0	39.0	1.014568
8.5	47.5	39.0	1.014568
12.0	50.1	38.1	0.991155
12.5	52.0	39.5	1.027575
18.0	63.5	45.5	1.183663
		35.52	**0.924037**

Group 1

Before Exposure mm	After Exposure mm	RE mm	RRG
10.5	46.0	35.5	0.923517
17.4	60.0	42.6	1.108221
10.0	44.0	34.0	0.884495
8.0	48.0	40.0	1.040583
14.0	60.5	46.5	1.209677
11.0	49.0	38.0	0.988554
13.1	50.0	36.9	0.959938
11.0	52.9	41.9	1.09001
13.0	51.3	38.3	0.996358
9.0	43.1	34.1	0.887097
		38.78	**1.008845**

2 mg/ml (continued)

Group 2				Group 3			
Before Exposure mm	After Exposure mm	RE mm	RRG	Before Exposure mm	After Exposure mm	RE mm	RRG
12.5	49.5	37.0	0.962539	14.0	46.1	32.1	0.835068
15.1	58.0	42.9	1.116025	16.5	58.5	42.0	1.092612
16.5	56.2	39.7	1.032778	13.5	47.5	34.0	0.884495
14.5	51.0	36.5	0.949532	10.0	43.2	33.2	0.863684
19.0	59.1	40.1	1.043184	9.0	45.0	36.0	0.936524
9.0	38.1	29.1	0.757024	10.5	51.0	40.5	1.05359
12.5	40.5	28.0	0.728408	14.0	40.6	26.6	0.691988
11.0	47.5	36.5	0.949532	13.2	53.0	39.8	1.03538
17.0	55.3	38.3	0.996358	11.0	49.5	38.5	1.001561
20.0	63.8	43.8	1.139438	16.4	55.0	38.6	1.004162
		37.19	**0.967482**			**36.13**	**0.939906**

(Note: the significant numbers in the calculation is two digits more than what is reported to get more accurate calculation results).

Statistical analysis using *one-way ANOVA* procedure:

Step 1: Comparison among the three groups in each concentration based on the RE values

I) 20 mg/ml

1) Calculation of *SST* and *SSE*

$$SST = \sum_{i=1}^{3} n_i (\bar{y}_i - \bar{y})^2$$

$$SSE = \sum_{i=1}^{3} \sum_{j=1}^{n_i} (y_{ij} - \bar{y}_i)^2$$

Here:

$n_1 = n_2 = n_3 = 10$, $\bar{y}_1 = 36.06$, $\bar{y}_2 = 35.09$, $\bar{y}_3 = 35.52$, and $\bar{y} = 35.56$, then

$$SST = 4.72,\ SSE = 1622.63$$

2) Calculation of *MST* and *MSE*

$$MST = SST / DFT$$

$$MSE = SSE / DFE$$

Here, $DFT = k - 1 = 2$, $DFE = (n_1 + n_2 + n_3) - k = 30 - 3 = 27$, then

$$MST = 2.36, MSE = 60.10$$

3) Calculation of F

$$F = MST / MSE = 2.36 / 60.10 = 0.039$$

4) Comparison between the calculated F and the tabular critical F

The degrees of freedom, $DFT = 2$, and $DFE = 27$, the tabular critical F with such degrees of freedom is 3.354 (www.itl.nist.gov, 2004). The calculated F, 0.039, is much smaller than the critical F. It indicates that statistically, the three groups of data that are being compared are not significantly different. The p value is calculated from the calculated F, DFT, and DFE, i.e., 0.039, 2, and 27 (graphpad.com/quickcalcs/PValue1.cfm, 2004). The p value is 0.96.

5) Summary – the *ANOVA* table

Source	SS	DF	MS	F
Treatment	4.72	2	2.36	0.039
Error	1622.63	27	60.10	
Total	1627.35	29		

II) 2 mg/ml

The analysis is performed based on the RE values, and is according to the above procedure from 1) to 5). The data used for the calculation are

$$n_1 = n_2 = n_3 = 10,\ \bar{y}_1 = 38.78,\ \bar{y}_2 = 37.19,\ \bar{y}_3 = 36.13$$

$$\bar{y} = 37.37,\ DFT = 2,\ DFE = 27.$$

The *ANOVA* table is:

Source	SS	DF	MS	F
Treatment	35.58	2	17.79	0.82
Error	587.43	27	21.76	
Total	623.01	29		

The tabular critical F with the degrees of freedom 2 and 27 is 3.354. The p value is determined to be 0.45. The statistical analysis based on the RE values of the *C. sativus* seedlings exposed to 2 mg/ml indicates that there is no significant difference exists among the three groups of data.

Step 2: Comparison between the mean RE values for the two concentrations

The comparison is done following the procedure that has been discussed from 1) to 5). Now there are two groups of data to be compared. The i in the calculation of *SST* and *SSE* is from 1 to 2. The data used for the calculation now is

$$n_1 = n_2 = 30,\ \bar{y}_1 = 35.56,\ \bar{y}_2 = 37.37$$

$$\bar{y} = 36.46,\ DFT = 1,\ DFE = 58.$$

The *ANOVA* table is:

Source	SS	DF	MS	F
Treatment	49.14	1	49.14	1.24
Error	2299.50	58	39.65	
Total	2348.64	59		

The tabular critical F value at the degrees of freedom 1 and 58 is 4.007. The calculated F, 1.24, is smaller than 4.007, indicating there is no statistically significant difference exists between the two mean RE values. The p is determined as 0.27.

The statistical result reported in this dissertation (see Table 4.11) was obtained based on the RE values for the three concentrations of 20 mg/ml, 2 mg/ml, and 200 μg/ml. The number of the groups was 3. $n_1 = n_2 = n_3 = 30$, $\bar{y}_1 = 35.56$, $\bar{y}_2 = 37.37$, $\bar{y}_3 = 38.17$ $\bar{y} = 37.030$, $DFT = 1$, and $DFE = 87$. The *ANOVA* table is:

Source	SS	DF	MS	F
Treatment	107.28	2	53.64	1.41
Error	3304.99	87	37.99	
Total	3412.27	89		

The tabular critical F at the degrees of freedom of 2 and 87 is 3.101. The p value is determined as 0.25.

APPENDIX B
THE MEAN RE VALUES

Note: the results are reported as mean RE ± standard deviation (S.D.). The significant numbers reported here for the mean RE values and the S.D. are one digit more than what was reported in the dissertation, which is to ensure the accuracy of the statistical analysis.

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
13-nm Alumina (24-hr Exposure)	Z. mays	Blank for 20 mg/ml	1	13.91 ± 3.99	10
			2	14.79 ± 5.04	10
			3	12.54 ± 4.90	10
		20 mg/ml	1	7.44 ± 1.18	10
			2	7.45 ± 1.43	10
			3	6.80 ± 1.82	10
		Blank for the other three concentrations	1	19.60 ± 4.36	10
			2	20.30 ± 8.33	10
			3	20.17 ± 5.34	10
		2 mg/ml	1	15.58 ± 4.20	10
			2	15.97 ± 4.64	10
			3	16.03 ± 3.10	10
		200 μg/ml	1	18.16 ± 3.76	10
			2	18.31 ± 2.60	10
			3	18.77 ± 3.84	10
		20 μg/ml	1	20.14 ± 4.94	10
			2	19.59 ± 1.55	10
			3	22.33 ± 4.52	10
	C. sativus	Blank for 2 mg/ml	1	24.65 ± 7.89	10
			2	27.35 ± 7.55	10
			3	28.88 ± 2.47	10
		Blank for the other three concentrations	1	56.36 ± 5.39	10
			2	49.15 ± 5.25	10
			3	51.49 ± 3.27	10
		20 mg/ml	1	26.83 ± 3.05	10
			2	26.21 ± 6.83	10
			3*	20.56 ± 7.00	10
		2 mg/ml	1	23.42 ± 3.50	10
			2	22.66 ± 4.91	10
			3	21.74 ± 2.77	10
		200 μg/ml	1	34.15 ± 7.11	10
			2	34.40 ± 9.43	10
			3	36.18 ± 6.50	9
		20 μg/ml	1	36.74 ± 3.13	10

* Discarded

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
		20 μg/ml	2	38.24 ± 5.67	10
13-nm Alumina	C. sativus		3	36.40 ± 5.86	10
(24-hr Exposure)	B. oleracea	Blank for 2 mg/ml	1	17.97 ± 5.49	10
			2	20.09 ± 2.13	10
			3	18.43 ± 2.98	10
		Blank for the other three concentrations	1	16.16 ± 4.19	10
			2	18.35 ± 3.54	10
			3	16.31 ± 8.20	9
		20 mg/ml	1	9.43 ± 2.55	10
			2	11.22 ± 3.98	10
			3	9.13 ± 3.91	10
		2 mg/ml	1	14.48 ± 1.55	10
			2	14.83 ± 3.34	10
			3	14.97 ± 2.29	10
		200 μg/ml	1	14.48 ± 6.23	10
			2	13.48 ± 5.63	10
			3	16.27 ± 2.27	9
		20 μg/ml	1	19.82 ± 7.74	10
			2	15.94 ± 7.09	10
			3	17.61 ± 7.28	10
	D. carota	Blank for 20 mg/ml	1	8.39 ± 1.57	10
			2	8.01 ± 1.39	10
			3	8.54 ± 2.07	10
		Blank for the other three concentrations	1	9.92 ± 2.79	10
			2	8.80 ± 1.88	10
			3	10.81 ± 2.12	10
		20 mg/ml	1	4.79 ± 1.20	10
			2	4.81 ± 1.35	10
			3	5.03 ± 1.61	10
		2 mg/ml	1	8.61 ± 1.29	10
			2	6.65 ± 2.51	9
			3	9.23 ± 1.94	10
		200 μg/ml	1	8.47 ± 5.51	10
			2	9.94 ± 2.56	10
			3	9.10 ± 2.30	10
		20 μg/ml	1	10.13 ± 3.19	10
			2	10.96 ± 3.07	10
			3	12.17 ± 2.12	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
13-nm Alumina	B. oleracea	Blank	1	16.16 ± 4.19	10
(48-hr Exposure)			2	16.36 ± 3.74	10
(1st 24-hr)			3	16.44 ± 4.88	10
		20 mg/ml	1	8.84 ± 2.59	10
			2	10.62 ± 3.98	10
			3	8.50 ± 3.82	10
13-nm Alumina	B. oleracea	Blank	1	27.62 ± 12.56	10
(48-hr Expo, 48-hr)			2	28.78 ± 8.48	10
			3	33.49 ± 8.75	10
		20 mg/ml	1	17.10 ± 3.25	10
			2	18.70 ± 5.24	10
			3	15.57 ± 7.34	10
13-nm Alumina	C. sativus	Blank	1	41.17 ± 4.64	10
(72-hr Exposure)			2	39.90 ± 5.39	10
(1st 24-hr)			3	36.22 ± 4.72	10
		2 mg/ml	1	34.57 ± 5.82	10
			2	35.45 ± 4.35	10
			3	36.18 ± 5.66	10
13-nm Alumina	C. sativus	Blank	1	71.69 ± 7.86	10
(72-hr Expo, 48-hr)			2	71.00 ± 16.12	10
			3	69.95 ± 13.01	10
		2 mg/ml	1	67.94 ± 9.80	10
			2	63.39 ± 13.07	10
			3	63.38 ± 9.34	10
13-nm Alumina	C. sativus	Blank	1	92.67 ± 10.39	10
(72-hr Expo, 72-hr)			2	95.11 ± 15.95	10
			3	94.79 ± 13.08	10
		2 mg/ml	1	87.31 ± 10.42	10
			2	86.63 ± 16.59	10
			3	88.74 ± 11.96	10
13-nm Alumina	C. sativus	Blank	1	47.19 ± 15.49	10
(Water treatment)			2	45.70 ± 12.41	10
(1st 24 hrs for expo.)			3	45.97 ± 10.62	10
		2 mg/ml	1	38.47 ± 6.63	10
			2	37.20 ± 7.37	10
			3	36.00 ± 9.74	10
13-nm Alumina	C. sativus	Blank	1	84.96 ± 15.83	10
(Water treatment)			2	81.29 ± 17.94	10
(48 hrs)			3	82.65 ± 11.44	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
13-nm Alumina	C. sativus	2 mg/ml	1	70.60 ± 14.00	10
(Water treatment)			2	66.85 ± 15.14	10
(48 hrs)			3	63.87 ± 18.15	10
14-nm Silica	B. oleracea	Blank	1	16.16 ± 4.19	10
(24-hr Exposure)			2	18.35 ± 3.54	10
			3	16.31 ± 8.20	9
		20 mg/ml	1	20.24 ± 5.46	10
			2	21.54 ± 5.52	10
			3	17.88 ± 3.38	10
		2 mg/ml	1	20.58 ± 4.99	10
			2	19.26 ± 6.20	10
			3	17.15 ± 7.72	10
		200 µg/ml	1	17.49 ± 6.65	10
			2	16.46 ± 4.96	10
			3	16.35 ± 6.82	10
		20 µg/ml	1	15.07 ± 4.10	10
			2	16.64 ± 3.58	10
			3	17.41 ± 4.93	9
	D. carota	Blank	1	12.88 ± 3.46	10
			2	11.06 ± 2.75	10
			3	14.59 ± 4.24	10
		20 mg/ml	1	13.78 ± 3.55	10
			2	14.48 ± 2.52	10
			3	16.02 ± 3.72	10
		2 mg/ml	1	12.50 ± 2.92	10
			2	11.65 ± 2.88	10
			3	14.33 ± 3.00	10
		200 µg/ml	1	13.32 ± 3.06	10
			2	12.09 ± 2.27	10
			3*	7.83 ± 2.35	10
		20 µg/ml	1	14.17 ± 4.83	10
			2	11.86 ± 3.52	9
			3	10.92 ± 2.43	10
	C. sativus	Blank	1	19.74 ± 5.20	10
			2	16.89 ± 6.11	10
			3	15.52 ± 6.33	8
		20 mg/ml	1	21.55 ± 6.62	10
			2	20.87 ± 6.92	10

* Discarded

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
14-nm Silica (24-hr Exposure)	C. sativus	20 mg/ml	3*	14.38 ± 5.59	10
		2 mg/ml	1	17.40 ± 5.69	10
			2	19.16 ± 9.60	10
			3*	13.56 ± 7.28	10
		200 µg/ml	1	17.30 ± 6.54	10
			2	19.61 ± 3.75	10
			3	18.40 ± 5.54	10
		20 µg/ml	1	17.70 ± 7.32	10
			2	17.87 ± 5.37	10
			3	19.28 ± 5.96	10
14-nm Silica (48-hr Exposure) (1st 24-hr)	B. oleracea	Blank	1	20.79 ± 5.48	10
			2	22.69 ± 6.45	10
			3	21.59 ± 9.30	10
		20 mg/ml	1	28.08 ± 4.99	10
			2	26.76 ± 5.02	10
			3	24.64 ± 6.33	10
14-nm Silica (48-hr Exposure) (48-hr)	B. oleracea	Blank	1	38.15 ± 10.48	10
			2	39.87 ± 7.42	10
			3	39.99 ± 12.04	10
		20 mg/ml	1	46.99 ± 9.21	10
			2	52.94 ± 11.52	10
			3	49.46 ± 11.52	10
14-nm Silica (Water treatment) (1st 24-hr Exposure)	B. oleracea	Blank	1	16.16 ± 4.19	10
			2	17.51 ± 4.58	10
			3	16.44 ± 7.74	10
		20 mg/ml	1	20.24 ± 5.46	10
			2	21.54 ± 5.52	10
			3	17.88 ± 3.38	10
14-nm Silica (Water treatment) (48-hr)		Blank	1	31.99 ± 11.10	8
			2	30.80 ± 6.76	7
			3	33.49 ± 8.75	8
		20 mg/ml	1	39.84 ± 11.20	10
			2	41.90 ± 11.17	10
			3	38.41 ± 7.30	10
21-nm Titania (24-hr Exposure)	B. oleracea	Blank	1	12.92 ± 4.91	10
			2	15.11 ± 4.29	10
			3	14.08 ± 3.84	10
		20 mg/ml	1	14.84 ± 3.07	8
			2	11.85 ± 4.34	10

* Discarded

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
21-nm Titania (24-hr Exposure)	B. oleracea	20 mg/ml	3	13.21 ± 2.18	10
		2 mg/ml	1	15.47 ± 4.85	10
			2	16.86 ± 3.50	10
			3	16.36 ± 3.38	10
		200 µg/ml	1	15.08 ± 2.10	10
			2	14.84 ± 3.90	10
			3	14.65 ± 4.24	9
	C. sativus	Blank	1	40.04 ± 5.39	10
			2	35.97 ± 4.11	10
			3	39.24 ± 3.27	10
		20 mg/ml	1	36.06 ± 7.73	10
			2	35.09 ± 5.94	10
			3	35.52 ± 9.24	10
		2 mg/ml	1	38.78 ± 4.02	10
			2	37.19 ± 5.20	10
			3	36.13 ± 4.70	10
		200 µg/ml	1	37.07 ± 7.21	10
			2	39.45 ± 5.19	10
			3	37.98 ± 5.92	10
	D. carota	Blank	1	8.39 ± 1.57	10
			2	7.98 ± 1.57	10
			3	8.40 ± 1.51	10
		20 mg/ml	1	7.80 ± 1.88	10
			2	6.78 ± 1.69	10
			3	7.78 ± 1.68	9
		2 mg/ml	1	7.60 ± 2.95	10
			2	7.32 ± 1.83	10
			3	7.32 ± 1.68	10
		200 µg/ml	1	9.31 ± 2.03	10
			2	9.20 ± 1.67	10
			3	9.24 ± 1.99	10
	A. sativa	Blank	1	13.68 ± 4.84	8
			2	11.85 ± 5.14	8
			3	14.21 ± 2.67	7
		20 mg/ml	1	12.30 ± 3.55	10
			2	13.22 ± 5.34	9
			3	12.27 ± 3.17	10
		2 mg/ml	1	14.10 ± 3.26	9
			2	11.48 ± 3.31	8

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
21-nm Titania (24-hr Exposure)	A. sativa	2 mg/ml	3	13.39 ± 4.93	9
		200 µg/ml	1	11.42 ± 3.44	7
			2	12.77 ± 3.95	10
			3	15.90 ± 3.21	8
21-nm Titania (48-hr Exposure) (1^{st} 24-hr)	C. sativus	Blank	1	40.04 ± 5.39	10
			2	36.48 ± 4.39	10
			3	38.55 ± 3.00	10
		20 mg/ml	1	36.06 ± 7.73	10
			2	35.09 ± 5.94	10
			3	35.52 ± 9.24	10
		2 mg/ml	1	38.78 ± 4.02	10
			2	37.19 ± 5.20	10
			3	36.13 ± 4.70	10
21-nm Titania (48-hr Exposure) (48-hr)	C. sativus	Blank	1	75.25 ± 17.61	10
			2	73.68 ± 10.81	10
			3	76.78 ± 16.79	10
		20 mg/ml	1	74.71 ± 14.36	9
			2	76.63 ± 15.65	10
			3	76.95 ± 12.15	10
		2 mg/ml	1	71.36 ± 6.92	9
			2	68.33 ± 6.40	10
			3	69.49 ± 5.97	8
161.2-nm Silica (24-hr Exposure)	B. oleracea	Blank	1	22.00 ± 5.39	9
			2	21.09 ± 5.93	9
			3	22.16 ± 6.55	9
		20 mg/ml	1	11.18 ± 1.94	9
			2	12.62 ± 3.55	8
			3	16.89 ± 4.49	8
		2 mg/ml	1	20.79 ± 4.93	10
			2	18.45 ± 7.53	10
			3	19.34 ± 7.55	10
		200 µg/ml	1	20.68 ±5.23	10
			2	21.54 ± 6.27	8
			3	26.76 ± 4.92	9
	C. sativus	Blank	1	30.76 ± 6.72	10
			2	31.72 ± 3.51	10
			3	33.18 ± 5.04	10
		20 mg/ml	1	24.78 ± 5.35	9
			2	24.71 ± 4.53	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
161.2-nm Silica (24-hr Exposure)	C. sativus	20 mg/ml	3	23.89 ± 3.75	10
		2 mg/ml	1	27.42 ± 6.09	10
			2	29.82 ± 5.21	10
			3	30.09 ± 3.82	10
		200 µg/ml	1	31.59 ± 4.82	10
			2	30.88 ± 3.68	9
			3	30.99 ± 4.62	10
	A. sativa	Blank	1	23.87 ± 4.82	10
			2	26.02 ± 2.07	10
			3	27.89 ± 3.94	10
		20 mg/ml	1	16.28 ± 5.80	8
			2	17.20 ± 4.84	8
			3	18.30 ± 6.37	9
		2 mg/ml	1	22.36 ± 5.27	10
			2	23.07 ± 1.11	10
			3	24.53 ± 4.58	10
		200 µg/ml	1	24.24 ± 3.24	10
			2	23.86 ± 0.70	10
			3	25.86 ± 3.39	10
161.2-nm Silica (48-hr Exposure) (1^{st} 24-hr)	B. oleracea	Blank	1	22.00 ± 5.39	9
			2	21.09 ± 5.93	9
			3	22.16 ± 6.55	9
		20 mg/ml	1	12.97 ± 6.58	10
			2	14.75 ± 7.06	10
			3	15.86 ± 6.48	10
161.2-nm Silica (48-hr Exposure) (48-hr)	B. oleracea	Blank	1	38.64 ± 9.19	7
			2	40.91 ± 7.41	8
			3	38.31 ± 5.90	8
		20 mg/ml	1	27.00 ± 7.87	7
			2	27.26 ± 8.04	9
			3	31.14 ± 13.16	7
161.2-nm Silica (Water treatment) (1^{st} 24-hr)	B. oleracea	Blank	1	22.00 ± 5.39	9
			2	21.09 ± 5.93	9
			3	22.16 ± 6.55	9
		20 mg/ml	1	13.26 ± 2.48	10
			2	13.43 ± 2.75	10
			3	14.47 ± 2.73	10
161.2-nm Silica (Water treatment) (48-hr)	B. oleracea	Blank	1	38.64 ± 9.19	7
			2	40.91 ± 7.41	8
			3	38.31 ± 5.90	8

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
161.2-nm Silica	B. oleracea	20 mg/ml	1	24.02 ± 7.60	10
(Water treatment)			2	25.52 ± 7.32	10
(48-hr)			3	25.77 ± 10.54	10
667.6-nm Silica	B. oleracea	Blank	1	23.33 ± 6.03	10
(24-hr Exposure)			2	24.17 ± 5.39	9
			3	19.82 ± 6.86	10
		20 mg/ml	1	22.55 ± 7.25	10
			2	18.91 ± 8.20	10
			3	24.16 ± 8.12	10
		2 mg/ml	1	23.09 ± 5.66	10
			2	18.39 ± 3.50	10
			3	22.73 ± 4.20	10
	C. sativus	Blank	1	32.17 ± 3.24	10
			2	32.76 ± 5.60	10
			3	34.20 ± 3.27	10
		20 mg/ml	1	35.13 ± 3.20	10
			2	33.22 ± 4.55	10
			3	32.97 ± 4.25	10
		2 mg/ml	1	31.15 ± 3.98	10
			2	30.05 ± 5.28	10
			3	31.79 ± 4.98	9
0.96-μm Titania	B. oleracea	Blank	1	23.33 ± 6.03	10
(24-hr Exposure)			2	20.72 ± 6.62	9
			3	22.92 ± 6.43	10
		20 mg/ml	1	20.80 ± 3.52	10
			2	19.29 ± 5.23	10
			3	20.26 ± 5.22	10
		2 mg/ml	1	20.29 ± 3.85	10
			2	24.40 ± 7.26	10
			3	24.09 ± 5.15	10
	C.sativus	Blank	1	32.17 ± 3.24	10
			2	32.76 ± 5.60	10
			3	34.20 ± 3.27	10
		20 mg/ml	1	32.84 ± 3.79	10
			2	32.91 ± 7.21	10
			3	30.74 ± 5.10	10
		2 mg/ml	1	34.20 ± 3.55	10
			2	33.44 ± 2.69	10
			3*	37.44 ± 4.16	10

* Discarded

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
1.00-μm Alumina (24-hr Exposure)	B. oleracea	Blank	1	15.92 ± 2.67	10
			2	16.22 ± 5.74	10
			3	14.62 ± 3.24	10
		20 mg/ml	1	14.48 ± 4.00	10
			2	15.03 ± 4.30	7
			3	15.30 ± 4.23	10
		2 mg/ml	1	14.91 ± 5.44	9
			2	15.25 ± 3.53	8
			3	15.76 ± 4.25	10
	C. sativus	Blank	1	32.17 ± 3.24	10
			2	32.76 ± 5.60	10
			3	34.20 ± 3.27	10
		20 mg/ml	1	32.07 ± 2.07	10
			2	30.99 ± 2.48	9
			3	33.64 ± 4.06	10
		2 mg/ml	1	33.18 ± 4.55	10
			2	32.07 ± 3.50	10
			3	32.73 ± 2.05	10
13-nm Alumina (Phen-loading)	C. sativus	Blank 1	1	24.65 ± 7.88	10
			2	27.35 ± 5.47	10
			3	28.88 ± 2.47	10
		Nonloaded (2 mg/ml)	1	23.42 ± 3.50	10
			2	22.66 ± 0.37	10
			3	21.74 ± 2.77	10
		10.0% MML (2 mg/ml)	1	27.11 ± 5.42	10
			2	29.26 ± 6.20	10
			3	28.71 ± 6.83	10
		Blank 2	1	19.60 ± 4.36	10
			2	20.30 ± 8.33	10
			3	20.17 ± 5.34	10
		100.0% MML (2 mg/ml)	1	18.05 ± 2.91	10
			2	20.89 ± 5.99	10
			3	19.13 ± 4.40	10
		432.4% MML (2 mg/ml)	1	19.15 ± 3.56	10
			2	16.79 ± 3.50	10
			3	19.22 ± 5.30	10
		Blank 3	1	23.57 ± 3.69	10
			2	26.18 ± 7.08	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
13-nm Alumina (Phen-loading)	C. sativus	Blank 3	3	23.82 ± 4.20	10
		Phenanthrene	1	25.52 ± 4.78	10
		(0.28 mg/ml)	2	26.86 ± 4.91	10
			3	25.61 ± 6.35	10
	Z. mays	Blank	1	19.60 ± 4.36	10
			2	20.30 ± 8.33	10
			3	20.17 ± 5.34	10
		Nonloaded	1	16.76 ± 3.80	10
		(2 mg/ml)	2	16.03 ± 2.61	10
			3	16.17 ± 1.41	10
		10.0% MML	1	18.90 ± 4.15	10
		(2 mg/ml)	2	20.34 ± 4.00	10
			3	20.18 ± 6.00	10
	G. max	Blank	1	36.37 ± 7.43	10
			2	38.89 ± 14.42	10
			3*	45.23 ± 10.40	10
		Nonloaded	1	30.21 ± 7.75	10
		(2 mg/ml)	2	31.10 ± 6.21	10
			3	33.23 ± 8.02	10
		10.0% MML	1	34.74 ± 12.08	10
		(2 mg/ml)	2	34.82 ± 13.30	10
			3	40.00 ± 4.62	10
	B. oleracea	Blank	1	18.03 ± 5.29	10
			2	19.49 ± 3.39	10
			3	19.04 ± 1.89	10
		Nonloaded	1	14.48 ± 1.55	10
		(2 mg/ml)	2	14.83 ± 3.34	10
			3	14.97 ± 2.29	10
		10.0% MML	1	19.38 ± 3.49	10
		(2 mg/ml)	2	18.48 ± 3.20	10
			3	19.09 ± 3.33	10
	D. carota	Blank	1	13.61 ± 0.97	10
			2	12.89 ± 1.93	10
			3*	18.36 ± 1.24	10
		Nonloaded	1	11.17 ± 2.37	10
		(2 mg/ml)	2	10.36 ± 1.12	10
			3	12.04 ± 6.96	9
		10.0% MML	1	13.00 ± 2.62	10
		(2 mg/ml)	2	13.26 ± 5.30	10

* Discarded

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
13-nm Alumina	D. carota	10.0% MML	3	13.75 ± 2.41	10
13-nm Alumina	Z. mays	Blank 1	1	16.61 ± 8.98	10
(DMSO)			2	19.02 ± 10.50	10
			3	23.41 ± 9.52	10
		0.5% DMSO	1	24.26 ± 8.65	10
			2	20.50 ± 4.35	10
			3	18.37 ± 7.48	10
		1.0% DMSO	1	23.02 ± 6.67	10
			2	19.30 ± 6.64	10
			3	22.03 ± 8.16	10
		Blank 2	1	16.68 ± 4.12	10
			2	17.38 ± 3.62	10
			3	19.61± 7.57	10
		0.5% DMSO treated	1	18.93 ± 3.30	10
			2	17.98 ± 6.26	10
			3	19.53 ± 5.53	10
		1.0% DMSO treated	1	18.93 ± 3.50	10
			2	17.96 ± 6.24	10
			3	15.07 ± 2.00	10
		Untreated	1	11.97 ± 2.09	10
			2	14.72 ± 3.66	10
			3	15.13 ± 4.10	10
21-nm Titania	B. oleracea	Blank for	1	16.16 ± 4.19	10
(Phen-loading)		freshly loaded	2	16.31 ± 8.20	9
			3	18.35 ± 3.54	10
		Freshly loaded	1	19.22 ± 3.75	10
		(2 mg/ml)	2	19.38 ± 3.83	10
			3	17.36 ± 4.41	10
		Blank	1	22.00 ± 5.39	9
			2	20.77 ± 5.68	10
			3	22.69 ± 6.79	8
		Aged loaded	1	15.87 ± 5.42	10
		(2 mg/ml)	2	13.03 ± 5.97	10
			3	14.77 ± 6.01	10
		Fresh Phen	1	23.18 ± 6.89	10
		(0.02 mg/ml)	2	20.98 ± 5.61	10
			3	19.48 ± 4.15	10
		Aged Phen	1	16.68 ± 4.02	8
		(0.02 mg/ml)	2	22.76 ± 4.80	8

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
21-nm Titania (Phen-loading)	B. oleracea	Aged Phen (0.02 mg/ml)	3	22.91 ± 5.23	7
		Blank for	1	12.92 ± 4.91	9
		Nonloaded	2	15.11 ± 4.29	8
			3	14.08 ± 3.84	9
		Nonloaded	1	15.47 ± 4.85	10
		(2 mg/ml)	2	16.86 ± 3.50	8
			3	16.36 ± 3.38	9
	C. sativus	Blank for	1	30.76 ± 6.72	10
		freshly loaded	2	31.72 ± 3.51	10
		and aged loaded	3	33.18 ± 5.04	10
		Freshly loaded	1	33.88 ± 2.92	10
		(2 mg/ml)	2	29.52 ± 6.12	10
			3	31.61 ± 3.36	10
		Aged loaded	1	22.49 ± 4.58	10
		(2 mg/ml)	2	24.14 ± 3.46	10
			3	21.72 ± 5.76	10
		Blank for	1	32.17 ± 3.24	10
		fresh Phen and	2	32.76 ± 5.60	10
		aged Phen	3	34.20 ± 3.27	10
		Fresh Phen	1	32.46 ± 3.19	10
		(0.02 mg/ml)	2	31.71 ± 1.98	10
			3	31.53 ± 3.96	10
		Aged Phen	1	33.25 ± 4.41	10
		(0.02 mg/ml)	2	32.17 ± 2.93	10
			3	31.19 ± 3.44	10
		Blank for	1	40.04 ± 5.39	10
		nonloaded	2	35.97 ± 4.11	10
			3	39.24 ± 3.27	10
		Nonloaded	1	38.78 ± 4.01	10
		(2 mg/ml)	2	37.19 ± 5.20	10
			3	36.13 ± 4.70	10
14-nm Silica (Phen-loading)	B. oleracea	Blank for	1	23.33 ± 6.03	10
		loaded	2	22.92 ± 6.43	10
			3	20.72 ± 6.62	9
		90.6% MML	1	17.34 ± 3.05	10
		(2 mg/ml)	2	19.69 ± 4.16	10
			3	19.30 ± 3.87	10
		Blank for nonloaded	1	16.16 ± 4.19	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
14-nm Silica (Phen-loading)	B. oleracea	Blank for nonloaded	2	16.31 ± 8.20	9
			3	18.35 ± 3.54	10
		Nonloaded (2 mg/ml)	1	20.58 ± 4.99	10
			2	19.26 ± 6.20	10
			3	17.15 ± 7.72	10
	D. carota	Blank for loaded	1	14.25 ± 1.31	10
			2	13.56 ± 2.56	10
			3	13.24 ± 1.61	10
		90.6% MML (2 mg/ml)	1	12.26 ± 1.18	9
			2	12.67 ± 1.42	9
			3	10.70 ± 1.48	10
		Blank for nonloaded	1	12.88 ± 3.46	10
			2	11.06 ± 2.75	10
			3	14.59 ± 4.24	10
		Nonloaded (2 mg/ml)	1	12.50 ± 2.92	10
			2	11.65 ± 2.88	10
			3	14.33 ± 3.00	10
161.2-nm Silica (Phen-loading)	B. oleracea	Blank	1	19.40 ± 5.90	10
			2	21.33 ± 4.64	10
			3	16.05 ± 5.72	10
		Nonloaded but acetone treated (20 mg/ml)	1	18.99 ± 5.18	9
			2	18.37 ± 4.36	9
			3	16.80 ± 5.56	10
		99.6% MML (20 mg/ml)	1	14.10 ± 3.11	9
			2	15.33 ± 4.27	10
			3	16.45 ± 5.18	10
667.6-nm Silica (Phen-loading	B. oleracea	Blank	1	23.33 ± 6.03	10
			2	24.17 ± 5.39	9
			3	19.82 ± 6.86	10
		Nonloaded but acetone treated (20 mg/ml)	1	23.59 ± 5.32	8
			2	22.90 ± 6.69	9
			3	23.42 ± 5.98	8
		Loaded (20 mg/ml)	1	23.44 ± 6.05	9
			2	19.20 ± 5.70	10
			3	22.02 ± 4.26	10
0.96-μm Titania (Phen-loading)	B. oleracea	Blank	1	23.33 ± 6.03	10
			2	24.17 ± 5.39	9
			3	19.82 ± 6.86	10

THE MEAN RE VALUES
(Continued)

Particle (Test)	Plant Species	Conc.	Group #	Mean RE ± S.D. mm	Sample Size
0.96-μm Titania (Phen-loading)	B. oleracea	Nonloaded but acetone treated (20 mg/ml)	1	21.99 ± 6.64	9
			2	22.16 ± 6.29	9
			3	21.70 ± 6.53	9
		97.9% MML (20 mg/ml)	1	22.42 ± 9.04	10
			2	24.10 ± 13.52	10
			3	24.09 ± 6.72	10
1.0-μm Alumna (Phen-loading)	B. oleracea	Blank	1	23.33 ± 6.03	10
			2	24.17 ± 5.39	9
			3	19.82 ± 6.86	10
		Nonloaded but acetone treated (20 mg/ml)	1	21.43 ± 8.98	9
			2	21.27 ± 8.34	9
			3	21.28 ± 7.52	9
		92.6% MML (20 mg/ml)	1	21.50 ± 6.10	10
			2	19.48 ± 7.18	10
			3	25.14 ± 4.37	10

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